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Mechanisms of Progestin–Stimulated Sperm Hypermotility in Two Teleosts: the Atlantic Croaker (*Micropogonias undulatus*) and the Southern Flounder (*Platylicthys lethigstomata*)

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by

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DEDICATION

To my wife, Nicole; without you, your love and your support none of this would have been possible.

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Mechanisms of Progestin–Stimulated Sperm Hypermotility in Two Teleosts: the Atlantic Croaker (*Micropogonias undulatus*) and the Southern Flounder (*Platylicthys lethigstomata*)

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The goal of this research was to examine the role of the novel membrane progestin receptor alpha (mPRα) in the stimulation of sperm hypermotility by the progestin 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) in two teleosts; the Atlantic croaker (*Micropogonias undulatus*) and the southern flounder (*Platylicthys lethigstomata*). In croaker, the expression, localization and hormonal regulation of mPRα in testis and sperm were investigated, as were the intracellular signaling pathways activated by 20β-S and mPRα to induce croaker sperm hypermotility. In flounder, stimulation of sperm hypermotility by 20β-S and binding of this steroid to flounder sperm membranes were examined. Finally, expression of mPRα was investigated in flounder testes and the expression and localization of this receptor in flounder testis and sperm was examined.

In croaker sperm, mPRα was expressed on the plasma membrane and localized to the midpiece. Expression of mPRα was also shown to be associated with high sperm motility and regulated by gonadotropin. The signaling pathways activated by 20β-S in croaker sperm were shown to involve activation of olfactory G-proteins (G_{olf}). Subsequent activation of membrane adenylyl cyclases was also demonstrated and shown to be necessary for 20β-S-stimulated cAMP production and 20β-S-induction of sperm hypermotility. Furthermore, co-immunoprecipitation studies show mPRα and G_{olf} physically associate with one another, establishing mPRα as the mediator of 20β-S actions in croaker sperm. Finally, evidence was obtained for progestin-stimulation of sperm hypermotility and the presence of mPRα on sperm membranes in another marine teleost species belonging to a different family, the southern flounder. In addition, mPRα was shown to be expressed on flounder sperm membranes and also localized to the sperm midpiece.

Results from the following studies support the hypothesis that mPRα is the mediator of 20βS-stimulated sperm hypermotility in croaker and is a likely intermediary in southern flounder. Furthermore, these data provide a plausible mechanism by which 20β-S and mPRα stimulate croaker sperm hypermotility. In addition, these results provide the first evidence of hormonal activation of G_{olf} proteins for any species. Finally, mPRα-mediated mechanisms to increase sperm motility are suggested to be evolutionarily conserved in teleosts since they also likely exist in a non-sciaenid species, the southern flounder.

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CHAPTER 1

INTRODUCTION

For decades, steroid hormones were thought to act primarily via a "classical", or genomic, mechanism involving the transcription of DNA and protein synthesis following binding of a steroid to an intracellular receptor belonging to the nuclear receptor superfamily. However, there are numerous reports suggesting alternative mechanisms of steroid action, which do not require transcription of target genes to elicit a physiological response (Falkenstein et al., 2000). These nongenomic actions of steroids also differ from genomic ones in that they are much more rapid, and in many cases, are thought to involve receptors located on the plasma membrane. Yet for decades, the identities of the receptors which mediate the rapid actions of steroid hormones have remained elusive.

A novel gene, unrelated to nuclear receptors and with the characteristics of a membrane steroid receptor was recently cloned from the ovary of a teleost, the spotted seatrout (*Cynoscion nebulosus*), along with homologous genes in multiple vertebrate species (Zhu et al., 2003a; Zhu et al., 2003b). This receptor, called mPRα, is specific for the teleost progestin 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) and has been shown to be a necessary intermediary in oocyte maturation in this species (Thomas et al., 2007; Zhu et al., 2003b). Activation of mPRα by 20β-S results in activation of inhibitory G-proteins (G_i) which decreases intracellular cAMP concentrations and release seatrout oocytes from meiotic arrest (Pace and Thomas, 2005; Zhu et al., 2003b). Additional reports have shown that mPRs are involved in oocyte maturation of other fish species and

Xenopus, and are important intermediaries in human myometrial function (Josefsberg Ben-Yehoshua et al., 2006; Karteris et al., 2006; Kazeto et al., 2005; Tokumoto et al., 2006). These studies reveal the wide-spread significance of mPRs in female reproductive tissues of different vertebrate species.

Rapid actions of progestins have also been demonstrated in male gametes of several of vertebrate species. In particular, progesterone has been shown to rapidly stimulate hypermotility and the acrosome reaction in mammalian sperm (Baldi et al., 1995; Luconi et al., 2004; Parinaud and Milhet, 1996; Sabeur et al., 1996; Sirivaidyapong et al., 1999; Therien and Manjunath, 2003). In teleosts, progestins also stimulate sperm hypermotility, but they do not induce the acrosome reaction since teleost sperm lack an acrosome. In spotted seatrout, and a closely related species, Atlantic croaker (*Micropogonias undulatus*), the endogenous progestin 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) has been shown to rapidly stimulate sperm hypermotility (Thomas, 2003). Furthermore, a 20β-S receptor has been biochemically characterized on seatrout and croaker sperm membranes (Thomas et al., 1997; Thomas et al., 2005). Although the identities of the receptors which mediate these processes remain unclear, several candidates have been suggested. In humans, the classical progesterone receptor as well as a novel putative membrane progesterone receptor cloned from porcine liver are both present on sperm, but are expressed on the sperm head and acrosome, suggesting a role in the acrosome reaction (Meyer et al., 1996; Shah et al., 2005). In croaker sperm, mPRα is suggested to be the mediator of 20β-S-induced hypermotility since preliminary investigations have shown that mPRα is present on sperm membranes and localized to the

sperm midpiece, which is the region that is essential for the regulation of sperm motility (Thomas et al., 2005). However, a complete characterization of the expression and regulation of mPRα in male croaker is lacking. Moreover, the signaling pathways activated by 20β-S binding to mPRα in croaker sperm are unknown. In contrast to seatrout oocytes, in which 20β-S decreases intracellular cAMP and activates G_i proteins, treatment of croaker sperm with 20β-S results in increases in both intracellular cAMP and Ca^{2+} concentrations (Thomas, 2003; Thomas et al., 2004). Thus, whether G-proteins are activated in croaker sperm by 20β-S, and more importantly, which type, is not known.

Finally, in teleosts, direct actions of progestins on sperm to stimulate sperm motility have only been demonstrated for three members of the family *Sciaenidae* (Thomas et al., 2006). In two other species; the Japanese eel (*Anguilla japonica*) and the masu salmon (*Oncorhynchus masou)*, a different mechanism of progestin regulation of sperm motility is proposed, where progestins act on the sperm duct to increase seminal fluid pH to capacitate sperm, giving them the potential for motility (Miura et al., 1991; Todo et al., 2000). This differs from the sciaenid model in that direct actions of progestins on sperm have not been shown, and classical progestin receptors are proposed to mediate these effects (Ikeuchi et al., 2001). Interestingly, preliminary evidence for the direct action of progestins on sperm to increase motility has been obtained for the southern flounder (*Platylicthys lethigstomata*) (Thomas et al., 2006). However, information on the characteristics of the receptors involved in this species is lacking.

OVERALL GOALS

The overall goal of this research was to test the hypothesis that mPRα is the mediator of progestin-stimulation of sperm hypermotility in the Atlantic croaker and is a potential mediator in southern flounder. In order to do this, three specific objectives were developed. The first was to characterize the expression, localization and hormonal regulation of mPRα in croaker testes and sperm. The second objective was to characterize the intracellular pathways activated by 20β-S and mPRα to stimulate sperm hypermotility in croaker. The third objective was to characterize progestin-stimulation of sperm hypermotility and the receptor which regulates it in a non-sciaenid, the southern flounder, in order to investigate the importance of this mechanism in teleost sperm function.

CHAPTER 2

EXPRESSION, LOCALIZATION AND HORMONAL REGULATION OF mPRα IN ATLANTIC CROAKER TESTIS AND SPERM

Summary

Although there is clear evidence that progestins exert rapid actions on vertebrate sperm to stimulate hypermotility and the acrosome reaction, the receptor(s) which mediate these processes remain unidentified. There is even less information on the expression of membrane progestin receptors in other testicular cell types. Therefore, the expression, localization and hormonal regulation of membrane progestin receptor alpha (mPRα) in Atlantic croaker testes and sperm were investigated in the present study. Immunocytochemical analyses of croaker testes showed the mPRα protein was expressed in both germ and interstitial cell compartments. Expression of mPRα mRNA and protein was observed in testes over a wide range of gonadal developmental stages. Incubation of testicular fragments with 15 IU/mL hCG for 18 hr caused an increase in mPRα expression. Sperm from fish with high motility was shown to be associated with high mPRα protein expression by western blot and immunocytochemical analyses. Furthermore, *in vivo* treatment of croaker with luteinizing hormone releasing hormone analog resulted in a significant increase in sperm motility, which was associated with an increase in mPRα protein, but not mRNA expression. Taken together, these findings suggest that mPRα is expressed throughout testicular development and is hormonally

regulated, although its physiological role in the testis is unknown. In sperm, however, mPRα expression is associated with sperm motility and is increased by gonadotropin, suggesting that sperm are important sites of mPRα expression and regulation, which is consistent with this receptor's suggested role in the regulation of progestin-stimulated sperm hypermotility.

Introduction

A novel cDNA encoding a putative membrane progestin receptor (mPRα) was recently cloned from the ovary of the spotted seatrout (*Cynoscion nebulosus*) and was shown to be an important intermediary in oocyte maturation in this species (Zhu et al., 2003b). Since this initial report, over 20 genes encoding three different mPR subtypes (α, β and γ) have been described in numerous vertebrate species. The seatrout and human mPRα have been shown to localize to the plasma membrane and bind progestins specifically to initiate rapid signaling pathways through activation of inhibitory G-proteins (G_i) (Karteris et al., 2006; Thomas et al., 2007). Furthermore, mPRα homologs, and other mPR subtypes have been described in several vertebrate species and have been shown to regulate a variety of physiological functions including oocyte maturation in other fish species and *Xenopus*, and functional progesterone withdrawal in the human myometrium (Josefsberg Ben-Yehoshua et al., 2006; Karteris et al., 2006; Tokumoto et al., 2006; Zhu et al., 2003a). Together, these findings suggest a widespread role of mPRα in the regulation of reproductive functions in female vertebrates.

There is also evidence that progestin receptors are present on the cell membranes of male reproductive tissues of vertebrates (Baldi et al., 1995; Baldi et al., 2002; Luconi et al., 2004; Thomas et al., 2005; Uhler et al., 1992). For example, progesterone rapidly stimulates both sperm hypermotility and the acrosome reaction (AR) in some mammalian species, suggesting the involvement of membrane progestin receptors (Baldi et al., 2002; Sabeur et al., 1996; Sirivaidyapong et al., 1999; Therien and Manjunath, 2003). Multiple candidates for the receptors which mediate these processes have been proposed, but the true identities of these receptors remain unresolved (Ho and Suarez, 2001; Luconi et al., 2004). In teleosts, whose sperm lack an acrosome, and do not undergo the AR, progestin stimulation of sperm motility has been shown in three species; the spotted seatrout, the Atlantic croaker (*Micropogonias undulatus*) and the red drum (*Sciaenops ocellatus*) (Thomas et al., 2006; Thomas et al., 2004), and preliminary evidence suggests it also occurs in the southern flounder (*Paralicthys lethigstomata*) (Thomas et al., 2006). In seatrout and croaker, specific binding of progestins and mPRα expression on sperm membranes has been shown (Thomas et al., 1997; Thomas et al., 2005; Zhu et al., 2003b). Furthermore, mPRα is localized to the midpiece of sperm in these species, which supports its suggested role in progestin-stimulated sperm hypermotility (Thomas et al., 2005). In croaker sperm, we have shown that stimulation of sperm motility by the endogenous progestin 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) is associated with activation of an olfactory-type G-protein (G_{olf}; Chapter 2). This activation of G_{olf} results in activation of membrane adenylyl cyclases (mACs) which increase intracellular cAMP concentrations and likely leads to increases in Ca^{2+} concentrations via activation of

cAMP-gated Ca^{2+} channels (Chapter 2). This study also demonstrated that G_{olf} proteins are localized to the sperm midpiece and physically associate with mPRα. Although these findings propose a mechanism by which 20β-S and mPRα directly regulate sperm motility, information on the hormonal regulation of mPRα and the relationship between mPRα abundance and sperm motility is lacking. Furthermore, it is not known whether mPRα is expressed in other testicular cell types and if it plays a role in other testicular functions.

The roles of progestins and their receptors in testicular development and spermatogenesis in vertebrates are poorly understood. In teleosts, the classical paradigm of hormonal control of spermatogenesis involves gonadotropin stimulation of androgen production to promote the progression of spermatogonia to fully mature spermatozoa (Schulz and Miura, 2002). Studies in several teleosts indicate that 11-ketotestosterone (11-KT) is the primary regulator of this process (Miura and Miura, 2003; Schulz and Miura, 2002). However, spermiogenesis and the final maturation of sperm have been shown to be a progestin-regulated process in several species. In the masu salmon (*Oncorhynchus masou*) and the Japanese eel (*Anguilla japonica*), increases in circulating levels of the endogenous progestin 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) occur during spermiogenesis (Miura et al., 1991; Miura et al., 1992). Furthermore, 17,20β-P has been shown to act on the sperm duct, probably through nuclear progestin receptors, to increase sperm duct pH, through HCO^{3-} production by carbonic anhydrase, which is required for final sperm maturation and the acquisition of sperm motility (Miura and Miura, 2003). This mechanism differs from the other model of progestin stimulation of

sperm motility described above, in that a direct action of progestins on sperm of these species has not been shown. In addition, nuclear PRs have been suggested to mediate 17,20β-P regulation of sperm by this mechanism (Miura and Miura, 2003), and to date the involvement of non-classical receptors has not been demonstrated.

There is currently little information regarding the role of mPRs in the testis for any species. Expression of mPRα mRNA has been shown in human testis (Zhu et al., 2003a) and expression of mPRα and mPRβ proteins have been demonstrated in mouse testis (Thomas, 2004). For teleosts, preliminary evidence for testicular expression of mPRα has been shown in seatrout and croaker (Thomas et al., 2005; Zhu et al., 2003b). However, a detailed characterization of testicular mPR expression for any species is lacking. In addition, despite the known localization and expression of mPRα in croaker sperm, the hormonal regulation of this receptor in these cells is not clear. Thus, in the present study, the expression, localization and hormonal regulation of mPRα in the testis and sperm of the Atlantic croaker was examined.

Materials and Methods

Chemicals

Luteinizing hormone releasing hormone analog (LHRHa; des-Gly^{10} ,[d-Ala^{6}]-LHRH 91-90 ethylamide) was purchased from Bachem (Torrance, CA). 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) was purchased from Steraloids (Newport, RI). All others chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Animals and tissue collection

Young-of-the-year croaker were collected by trawl near Redfish Bay, Texas and transferred to tanks at the University of Texas Marine Science Institute. Fish were maintained in the laboratory for at least 1 month in 12,000 L recirculating saltwater tanks at 22-25ºC with a 11L:13D photoperiod to promote and maintain gonadal development. Sperm were collected using a syringe by applying gentle pressure to the abdominal region and avoiding the collection of urine which causes premature activation of sperm. For experiments on testicular tissue, croaker were deeply anesthetized and sacrificed by cervical dislocation following procedures approved by the Institutional Animal Care and Use Committee of University of Texas at Austin.

Membrane Preparation

Sperm and testicular plasma membranes were prepared as described previously (Thomas et al., 1997). Briefly, 2-3 ml of milt was pooled form multiple donors (n=3-5) or 1-2 g of testis was homogenized in cold homogenization buffer (HAED; 25 mM HEPES, 10 mM NaCl, 10 mM $MgCl_2$, 1 mM dithioerythritol, 1 mM EDTA, pH 7.6) with a glass homogenizer. Samples were then sonicated at a medium power on ice for 6 sec. Homogenates were then centrifuged at 500 x *g* for 20 min at 4ºC to remove the nuclear fraction. The resulting supernatants were collected and centrifuged at 17,000 x *g* for 30 min at 4ºC to obtain the plasma membrane fraction. Final membrane pellets were resuspended in HAED with HALT protease inhibitor cocktail (Pierce, Rockford, IL) to a

final protein concentration of ~1 mg/ml which was determined using a Bradford protein assay (Bio-Rad, Hercules, CA).

Western blot analyses

Approximately 10 μg of total membrane protein was resolved on 10% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked in 5% nonfat dry milk, 0.1% Tween 20 in phosphate buffered saline (PBS; 136 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 10 mM KH_2PO_4, pH 7.4). After three rinses with PBS, membranes were incubated with a rabbit polyclonal antibody which recognizes seatrout mPRα overnight in blocking solution at 4ºC (1:2000). This antibody is directed against a peptide sequence (YRQPDQSWRYYFLTL) in the N-terminal region of seatrout mPRα, which is identical in Atlantic croaker (Thomas et al., 2007) (Pace and Thomas, *unpublished obs.*). Membranes were then rinsed three times with PBS and incubated with goat anti-rabbit horseradish peroxidase-linked secondary antibody for 1 hr at room temperature (1:5000; Abcam, Cambridge, UK). Protein bands were visualized using SuperSignal West Pico chemilumenescent substrate (Pierce, Rockford, IL).

Immunolocalization

Croaker testes were collected and immediately frozen in liquid nitrogen. Testicular fragments were then embedded in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC), cryosectioned and mounted on slides coated with bioadhesive (Electron Microscopy Sciences, Hatfield, PA). Sections were then fixed in

methanol, rinsed with PBS, and blocked in 2% BSA in PBS for 2 hr. Slides were rinsed with PBS and incubated with the seatrout mPRα antibody directed against mPRα in blocking solution (1:2000) overnight at 4ºC. As a negative control, antisera were pre-absorbed against peptide (0.02 mg peptide/1 ml antibody) for 12 hr at 4ºC. Slides were rinsed three times with PBS and then incubated with a goat anti-rabbit AlexaFluor 488 secondary antibody (1:5000) for 2 hr at room temperature. After three rinses with PBS slides were incubated with 300 nM 4′,6-diamidino-2-phenylindole (DAPI) for 10 min, given a final rinse in PBS and coverslips added using ProLong Gold Antifade Reagent (Molecular Probes, Eugene, OR).

Sperm were collected and diluted 1000-fold in PBS and spread on poly-L-lysine coated slides. Samples were air dried for 30 min and then fixed in 4% paraformaldehyde on ice for 20 min. Slides were incubated with 13 mM $NaBH_4$ in PBS for 15 min to reduce autofluorescence and immunolocalization of mPRα performed as described above. Testis sections and sperm were examined using a Nikon Eclipse E600 fluorescent microscope.

Isolation of testicular interstitial cells

Testes were collected and immediately placed in calcium-magnesium free balanced salt solution (CMF-BSS: 138mM NaCl, 8.6mM KCl, 10mM $NaHCO_3$, 1.3mM Na_2HPO_4, 5mM D-glucose, 15mM Hepes, pH 7.4). Isolation and culture of testicular interstitial cells was performed following a protocol form Miura *et al*. (1996) with modifications. Testes were minced into fragments and enzymatically digested using 0.1% collagenase in CMF-BSS at room temperature for 2 hr. Following digestion, cell

suspensions were filtered through 40 μm nylon mesh to remove large tissue fragments and centrifuged at 500 x *g* to pellet cells. Pellets were resuspended in Dulbecco's Modified Eagle's Medium (DMEM, pH 7.2) with 100 mg/L streptomycin and 70 mg/L penicillin and then layered above equal volumes of 40% and 80% Percoll in DMEM and centrifuged at 680 x *g* for 20 min to remove erythrocytes. Cells at the boundaries of both the 40% and 80% Percoll layers were aspirated and centrifuged at 500 x *g*. Final cell pellets were resuspended in DMEM and incubated in culture plates with cell culture treated coverslips at 22ºC overnight. Using this protocol, interstitial and Leydig cells adhere to and grow on the bottom of cell culture plates (Miura et al., 1996). Localization of mPRα in testicular interstitial cells grown on coverslips was performed following the protocol described above. To confirm the presence of Leydig cells in insterstitial cell fractions, cell masses grown on coverslips were stained for 3β-hydroxysteroid dehydrogenase (3β-HSD) as described previously using nitro blue tetrazolium and 100 μg/ml dihydroepiandrosterone (DHEA) as a substrate (Benninghoff and Thomas, 2006).

Quantitative PCR

Total RNA was extracted from sperm and testes using TriReagent (Sigma, St. Louis, MO) following manufacturer's instructions. RNA was treated with a DNA Free RNA Kit (Zymo Research, Orange, CA) to remove genomic DNA. Complementary DNA was generated from approximately 500 ng of RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random primers. A set of reactions run without reverse transcriptase was used to control for genomic contamination.

Complementary DNA from croaker sperm and testes was then used to determine relative mPRα concentrations using a Brilliant SYBR Green QPCR kit following manufacturer's instructions (Stratagene, La Jolla, CA). The primers used for detection of a 300 bp fragment of mPRα were; sense-5'-GCTGGCGCTACTACTTTCTCA-3', antisense-5'-GGCAGCAGAAATAGGCG-3'. Relative mPRα concnetration was normalized to expression of a 200 bp region of the croaker 18S rRNA gene, which was detected using the following primers; sense 5'-GTTAATTCCGATAACGAACGAGACTC-3'; antisense 5'-ACAGACCTGTTATTGCTCAATCTCGTG-3'. For both primer sets a cycling profile of 10 min at 95ºC, 40 cycles of 30 sec at 95ºC, 1 min at 60ºC and 1 min at 72ºC was used. Relative mPRα expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Histology and assessment of reproductive stage

Testes were removed from croaker at different stages of gonadal development. All fish were undergoing early gonadal development or were fully developed. Gonadosomatic indices (GSIs) were calculated by dividing the mass of the gonad by total body mass. A small portion of testis was preserved in 10% neutral buffered formalin and sent to a commercial vendor for sectioning and hematoxylin and eosin staining (Pacific Pathology, San Diego, CA). Additional testicular fragments were frozen in liquid nitrogen for use in western blot and QPCR analyses described above. The reproductive stage of male Atlantic croaker was assessed by determining the percentages of germ cell cysts containing each of the four spermatogenic cell types (spermatogonia,

spermatocytes, spermatids and spermatozoa) surrounding three randomly selected seminiferous tubules from a single testicular section.

Sperm motility analyses

Sperm motility analyses were performed as described previously (Thomas and Doughty, 2004). Sperm were diluted 100-fold in predilution solution (160 mM NaCl, 8.6 mM KCl, 0.1 mM $CaCl_2$, 10 mM $NaHCO_3$, 1.3 mM Na_2HPO_4, 5 mM D-glucose, pH 7.8), and then treated with 20β-S (20 nM final concentration) or vehicle (ethanol, 0.02%) for 1 min. A 2 μL aliquot of each sperm suspension was then placed on a microscope slide and diluted with 25 μL of artificial seawater (680 mOs/kg). A coverslip was placed on the slide and viewed using dark field microscopy. Each experiment was recorded using a CCD camera (Cohu Electronics, San Diego, CA) and a VHS recorder to calculate the percentage of sperm displaying rapid turning and high velocity which are characteristic of hypermotility.

***In vitro* hormonal regulation of mPRα in croaker testes**

Testes from fully mature croaker were collected and placed immediately in DMEM with 100 mg/L streptomycin and 70 mg/L penicillin on ice. Testes were cut into small fragments weighing approximately 100 mg and incubated in DMEM with or without (control) 15 IU/mL human chorionic gonadotropin (hCG). At various time points (0, 1, 2, 4, 8, 12, 18 and 24 hr) fragments were removed and immediately frozen in liquid nitrogen. Concnetrations of mPRα protein were determined by western blot analyses and

semi-quantitative densitometry, and changes in mPRα abundance were calculated by dividing the expression of mPRα protein in hCG-treated samples by mPRα protein expression in control samples. Densitometry was performed using NIH ImageJ (v1.26) and a standard croaker testis sample was run on each gel to normalize relative mPRα protein expression across samples.

In vivo hormonal regulation of mPRα in croaker sperm

Croaker at early stages of testicular development, which were producing low quantities of sperm, were divided into groups of three and sperm from each group was collected and pooled. An aliquot of sperm was placed on ice and used for sperm motility analyses. The remainder of the sperm was frozen in liquid nitrogen. Groups of croaker were then given an intraperitoneal injection of 100 μg/kg of LHRHa (n=6) in 0.8% saline or saline alone (control groups). After 24 hr, sperm were collected, and sperm motility analyses were performed immediately. The remainder of the sperm was frozen for analysis of mPRα expression. Concnetrations of mPRα protein and mRNA in croaker sperm before and after LHRHa treatment were determined by western blot analyses with semi-quantitative densitometry or QPCR as described above.

Statistical analyses

All data are presented as means ± SEM. Statistical significance was determined using GraphPad Prism Software (San Diego, CA).

Results

Immunolocalization of mPRα in croaker testes

Immunohistochemical localization studies show that mPRα is widely expressed throughout the testis. Labeling of mPRα occurred in both interstitial and germ cell compartments with spermatogonia, spermatocysts and spermatids showing moderate expression of mPRα protein (Fig 2.1A-C). Immunolabeling of mPRα was most intense in the interstitial regions between seminiferous tubules and in germ cell cysts containing mature spermatozoa, which are identified by the alignment of all cells along the cyst wall in a semi-circular shape (Fig. 2.1A). The specificity of this reaction was confirmed by pre-incubating the mPRα antibody with the peptide antigen, which resulted in no labeling of testicular sections (Fig. 2.1D). Immunocytochemical experiments using isolated interstitial cells demonstrate that these cells also express mPRα, corroborating data from whole testes (Fig. 2.2A-D). Finally, histochemical staining of these cell masses for confirmed 3β-HSD activity resulted in the formation of black precipitates following incubation with DHEA in cell masses confirming the presence of steroidogenic Leydig cells (Fig. 2.2E, F).

Expression of mPRα in croaker testes throughout spermatogenesis

Testes of 13 croaker with various GSI's were examined histologically to determine their reproductive stage (Fig. 2.3A). The GSIs of these fish ranged from 0.01-9.04 and all but two fish had at least some germ cell cysts containing spermatozoa or spermatozoa present in the lumens of the seminiferous tubules. The percentage of germ

cell cysts at this spermatogenic stage ranged from 1% to approximately 30%. Expression of mPRα mRNA and protein was detected in all but two fish examined (Fig. 2.3B), but there was no clear relationship between mPRα expression and GSI, or percentage of any of the 4 germ cell stages examined.

Abundance of mPRα in croaker sperm and its association with sperm motility

To determine if mPRα abundance is associated with sperm motility, sperm was collected from individuals with low milt production (n=3), which is usually associated with low sperm motility and compared to fish with a high milt production (n=3), which normally display a high percentage of hypermotile sperm. In the present study, low milting groups had a mean control hypermotility of 70.2 ± 4.9 % compared to 80.68 ± 1.1 % in high motility groups. High motility groups also had a significantly higher mean percentage of hypermotile sperm in response to 20 nM 20β-S (88.6 ± 1.0 %) than low motility groups (82.4 ± 3.4 %) (Fig 2.4A). Western blot analyses of these samples demonstrated that expression of mPRα protein is greater in individuals with a relatively higher percentage of motile sperm (Fig. 2.4A). The abundance of mPRα, as measured by semi-quantitative densitometry, showed that the highly motile sperm had approximately 33% higher mPRα concentrations than low motility sperm donors (Fig. 2.4B). Immunoctyochemical experiments further support these findings, as mPRα labeling intensity of the midpiece and flagella of high motility sperm was greater than the intensity observed with low motility sperm incubated and visualized under the same conditions (Fig 2.5A, B).

In vitro hormonal regulation of mPRα in croaker testes

Testicular fragments from 5 croaker were incubated *in vitro* with hCG for various periods. However, testes from one fish did not respond to this treatment and were excluded from subsequent analyses. For the other fish, treatment of croaker testicular fragments with 15 IU/mL hCG resulted in a significant increase in mPRα protein abundance over the time course examined (Fig. 2.6). The magnitude of this increase was greatest after 18 hr incubation with hCG, which resulted in a 2-fold increase over control samples not receiving hCG. For each fish tested, there was a biphasic response in mPRα protein expression. This trend is reflected in Fig. 2.6, by the small increase in mPRα abundance at 2 hr and another, larger increase at 18 hr.

In vivo hormonal regulation of mPRα in croaker sperm

Treatment of croaker (n=6 groups) with 100 μg/kg LHRHa for 24 hr resulted in a significant increase in both basal and 20β-S-induced sperm motility (Fig. 2.7). This increase in sperm motility was associated with an increase in mPRα protein concentrations in croaker sperm membranes (Fig. 2.7). However, treatment of croaker with 100 μg/kg LHRHa did not result in a significant change in mPRα mRNA abundance (n=5 groups; Fig. 2.8). In both experiments, control groups receiving only an injection of saline showed no overall change in mPRα mRNA (n=5 groups) or protein (n=6 groups) abundance.

Discussion

The present results provide the first evidence in any vertebrate species that a member of the mPR family is ubiquitously expressed in testicular somatic and germ cells throughout spermatogenesis, suggesting it mediates diverse progestin actions in the vertebrate testis. The finding that mPRα expression in croaker testis and sperm is hormonally regulated and that elevated sperm receptor expression is associated with increased sperm hypermotility further indicate important physiological roles for mPRs in regulating testicular and sperm functions. Moreover, these results are consistent with previous reports suggesting that mPRα is the mediator of progestin-stimulation of sperm hypermotility in this species.

Little is known about the role of progestins in testicular function for any species. In contrast to the present findings in croaker, in which intermediate germ cell stages expressed mPRα, nuclear PR expression has been shown only in spermatogonia in boars and rats (Kohler et al., 2007; Weber et al., 2002). However, progesterone binding has been described in rat spermatocytes and spermatids (Galena et al., 1974), suggesting the presence of non-nuclear PRs in at least some male germ cell stages. In rat Leydig cells, progestin binding to plasma membranes has been described (Rossato et al., 1999). Furthermore, non-classical actions of progesterone have been suggested in two different mouse Leydig tumor cell lines, but the identities of the receptors which mediate these actions remain unresolved. In MA-10 cells, which lack nuclear PRs, micromolar concentrations of progesterone increase expression of steroidogenic acute regulatory protein (StAR), the rate-limiting enzyme controlling steroidogenesis (Schwarzenbach et

al., 2003). In the mLTC-1 cell line, similar concentrations of progesterone rapidly increase intracellular Ca^{2+}concentrations and decrease LH receptor expression (El-Hefnawy et al., 2000). Although the amounts of progesterone used in these studies are high, the authors propose that *in vivo* testicular concentrations of progesterone in the testis may approach micromolar concentrations, as testicular progesterone concentrations have been shown to be higher than circulating levels (Huhtaniemi et al., 1984). Interestingly, members of the mPR family are expressed in the testes of mice and humans (Thomas, 2004; Zhu et al., 2003a), although information on which cells they are expressed in is lacking. In light of the present findings in croaker, in which mPRα is expressed in germ cells and steroidogenic Leydig cells, it is possible that mPRs are expressed in testes of a wide range of vertebrate species and are important mediators of progestin actions in these tissues, such as regulation of steroidogenesis.

In teleosts, recent studies are beginning to elucidate the roles of progestins in testicular function. In particular, studies in the Japanese eel have suggested that progestins are important during early spermatogenesis, and are necessary mediators of the progression of meiosis in male germ cells (Miura et al., 2006). Although the receptors which mediate these effects are not known, the authors demonstrate that both teleost nuclear progestin receptors, PR I and PR II, are present in the eel testis with PR I expressed in germ cells, Sertoli cells and interstitial cells, while PR II expression restricted to germ cells. However, a direct link between these processes and nuclear PRs has not been established. The finding that mPRα is expressed in all germ cell types in croaker, including pre-meiotic spermatogonia and post-meiotic spermatocytes and

spermatids, suggests it may be involved in early actions of progestins during spermatogenesis. Moreover, the observation that all but two fish in this study possess at least some fully mature sperm in their testes, and that mPRα mRNA and protein were detected in testicular fragments from each fish examined, demonstrates that some degree of spermatogenesis is completed early in testicular development and further suggests that mPRα is a likely intermediary in this process.

The role of progestins in sperm function has received much more attention compared to their role in testicular function. In teleosts, there are two models regarding the role of progestins in the regulation of sperm motility. One model suggests that progestins act through classical PRs to increase sperm duct pH, to capacitate spermatozoa prior to their release (Ikeuchi et al., 2001; Miura et al., 1991; Miura et al., 1992; Todo et al., 2000). The other model proposes that progestins act on sperm directly, through non-classical progestin receptors, to rapidly increase intracellular cAMP concentrations to induce hypermotility. The latter model has also been suggested in a number of mammalian species (Baldi et al., 1995; Baldi et al., 2002; Uhler et al., 1992). Although the receptors on sperm which mediate progestin stimulation of sperm hypermotility are unknown, one candidate is mPRα, which has previously been shown to be expressed in sperm membranes of croaker. A recent study showed that 20β-S activates olfactory-type G-proteins to stimulate sperm motility in croaker (Chapter 2). Furthermore, mPRα co-localizes and physically associates with G_{olf} protein in croaker sperm. In the present study, the finding that concentrations of mPRα is higher in sperm with both higher basal motility as well as higher 20β-S-stimulated hypermotility strongly supports the

hypothesis that mPRα is the receptor which mediates progestin-stimulation of sperm motility. These findings suggest that mPRα abundance could be used as an indicator of sperm function. Receptor abundance has been proposed as an indicator of sperm function for classical PRs in humans in which PR expression was shown to be correlated with the ability of sperm to undergo the AR (Gadkar et al., 2002). Since PR is located to the acrosome region of human sperm, it likely plays a role in the stimulation of this process by progesterone (Shah et al., 2005). However, in teleost sperm, which lack an acrosome and do not undergo the AR, fertilization success is dependent upon the ability of sperm to enter a specialized pore in the egg called the micropyle. This requires that sperm have a high degree of motility. Thus, the level of expression of mPR in a population of sperm has potential to serve as a proxy for motility as well as an indicator of fertilization success. Such an approach might not be restricted to teleost sperm, since a positive correlation between mPRα expression and percent motile sperm has been demonstrated in spotted seatrout and human sperm (Tubbs and Thomas, *in press*) (Tubbs and Thomas, 2006).

The gonadotropin-induced increase in mPRα concetration in both croaker testes and sperm supports previous reports in seatrout and croaker in which the same treatment has been shown to elicit a 2-3-fold increase in 20β-S binding to testicular membranes (Thomas et al., 1997; Thomas et al., 2005), and suggests that mPRα is the progestin receptor that is upregulated under these conditions. Furthermore, *in vivo* treatment of croaker with LHRHa resulted in an increase in mPRα protein on sperm, which was associated with an increase in both basal sperm motility and progestin-stimulated sperm

motility. This association further suggests that mPRα is an important intermediary in progestin-stimulated sperm motility. Previous work in our laboratory has established that *in vivo* LHRHa treatment also results in an increase in plasma 20β-S concentrations (C. Detweiler, *unpublished thesis*) and together with the present findings provides a plausible mechanism to explain the observed low croaker sperm motility during the early and late portions of the reproductive season, when circulating levels of gonadotropin and steroids are low. Exactly how mPRα protein expression is increased by LHRHa treatment, however, is not clear. Sperm are widely considered to be transcriptionally inactive (Hecht, 1998), and lack the ability to synthesize RNA. Regardless, mRNA is present in sperm which are not translationally dormant and can synthesize new protein (Hecht, 1998; Miller and Ostermeier, 2006; Miller et al., 2005). The change in mPRα protein, but not mRNA expression, observed in croaker sperm in response to LHRHa is thus likely due to increased translational efficiency of mPRα mRNA, and similar findings have been reported in croaker and goldfish ovaries in response to gonadotropin (Tokumoto et al., 2006) (Pace and Thomas, *unpublished obs*.).

In teleosts, three mPR isoforms (α, β, and γ) have been discovered and may also be present in croaker sperm and testes. Zebrafish (*Danio rerio*) possess an mPRα and mPRβ (Zhu et al., 2003a; Zhu et al., 2003b), both of which show high mRNA expression in the testis. The channel catfish (*Ictalurus punctatus*), however, has mPRα, mPRβ and an mPRγ isoform (Kazeto et al., 2005). Like zebrafish, catfish mPRα and mPRβ mRNAs are highly expressed in the testis, whereas mPRγ shows little testicular expression. Furthermore, in mice, both mPRα and mPRβ protein have been shown to be expressed in

the testis (Thomas, 2004). Preliminary studies with human sperm in our laboratory suggest that mPRα, but not mPRβ, is expressed in mature germ cells (Thomas and Tubbs, *unpublished obs*.). Although mPRα is clearly shown to be expressed in croaker sperm and testes, the expression of other mPR subtypes, and what physiological roles they may play in these tissues warrant investigation.

In conclusion, the present study demonstrates the expression of the novel mPRα in the sperm and testes of the Atlantic croaker. In the testis, mPRα was shown to be expressed in both germ and interstitial cells and throughout testicular development. Furthermore, it appears to play a role in the development of the testis as it is regulated *in vitro* by gonadotropin and found in fish at all stages of development. In croaker sperm, expression of mPRα is localized to the midpiece and flagella, which is consistent with its suggested role in progestin-stimulation of sperm motility in this species. Moreover, populations of sperm with higher motility display higher mPRα concentrations. Treatment of croaker with LHRHa *in vivo* results in an increase in sperm motility as well as increased mPRα protein expression. These findings provide the first evidence of testicular expression of a mPR for any species and suggest that it is likely involved in the development and function of this tissue. Moreover, this study provides additional support for the suggested role of mPRα in progestin-stimulated sperm motility in the Atlantic croaker.

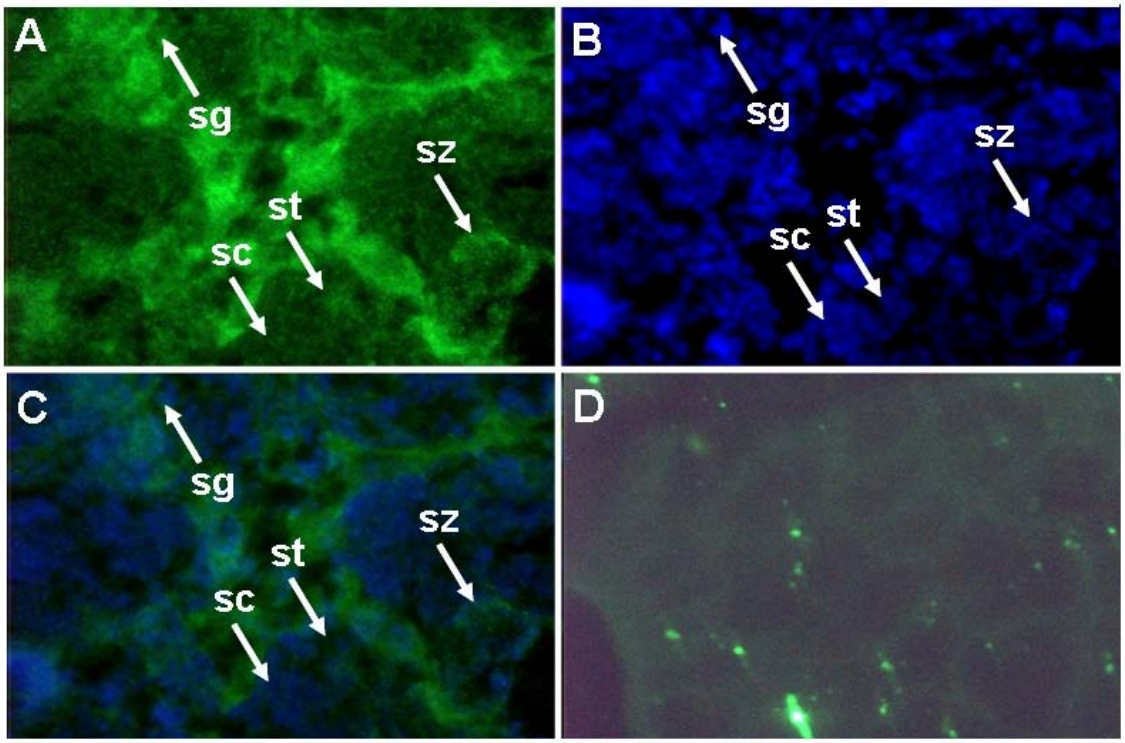

Figure 2.1. Immunohistochemical localization of mPRα in Atlantic croaker testes. Croaker testes were cryosectioned and incubated with a primary antibody directed against (A) seatrout mPRα (1:1000) and an AlexaFluor 488 secondary antibody (1:2000) to show the expression pattern of mPRα (green). (B) Cell nuclei (blue) were counterstained with DAPI. Germ cell cysts containing spermatogonia (sg), spermatocytes (sc), spermatids (st) and mature spermatozoa (sz) are shown. (C) Merged image of mPRα and DAPI labeling. (D) Experiments were performed after preabsorbing the mPRα antibody with the peptide antigen (0.02 mg peptide/ml antibody) to confirm antibody specificity. Results are typical of three experiments.

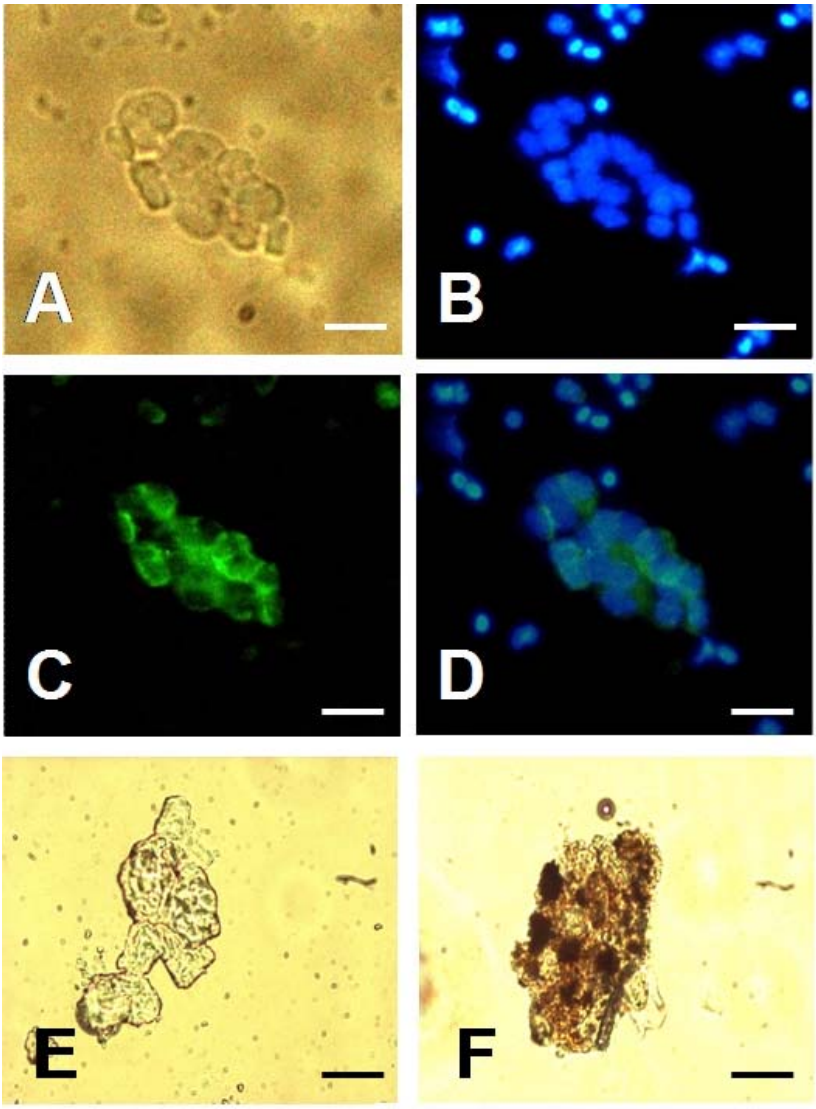

Fig. 2.2. Immunocytochemical localization of mPRα in Atlantic croaker interstitial and Leydig cells. Croaker testes were enzymatically digested and cultured overnight to isolate Sertoli and germ cells from Leydig and interstitial cells. Cells were grown on coverslips and incubated with an antibody directed against mPRα (1:1000) and an AlexaFluor 488 secondary antibody (1:2000). (A) Phase contrast image of Leydig and interstitial cell primary co-cultures. (B) Cell nuclei counterstained with DAPI. (C) Immunocytochemical

localization of mPRα protein. (D) Composite image of cell nuclei and mPRα labeling. (E-F) Confirmation of presence of Leydig cells by assessment of 3β-hydroxysteroid dehydrogenase activity in isolated testicular interstitial cells from croaker. Cells were incubated in the absence (E; control) or presence (F) of 0.25 mM nitro blue tetrazolium and 100 μg/ml dihydroepiandrosterone (DHEA). Formation of black precipitates in DHEA-treated cell masses reveals 3β-HSD activity. Bars represent 10 μm. Results are typical of four experiments.

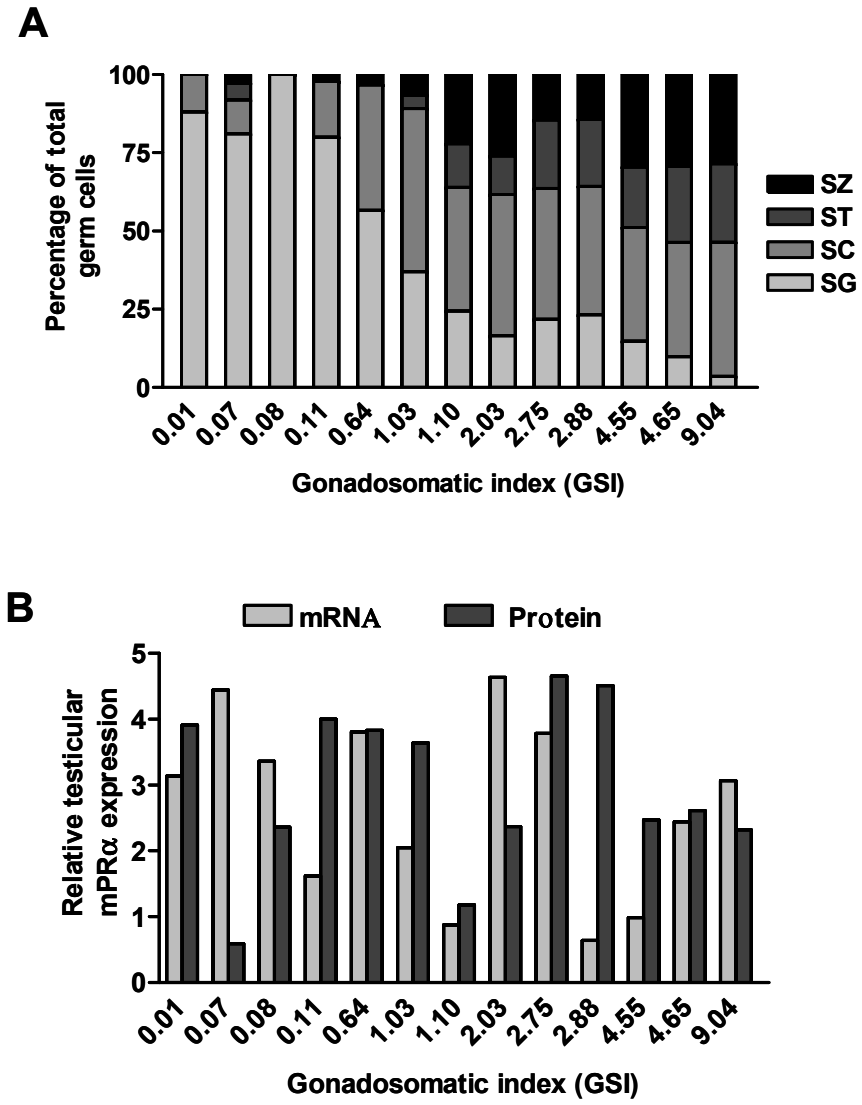

Figure 2.3. Expression of mPRα mRNA and protein in Atlantic croaker testes from fish at different stages of gonadal development. (A) Testes were collected from croaker of various gonadosomatic indices (GSI's) and reproductive stage of each fish was assessed by determining the percentage of germ cell cysts containing each of the 4 spermatogenic cell types surrounding 3 randomly selected seminiferous tubules (sg, spermatogonia; sc, spermatocytes; st, spermatids; sz, spermatozoa). (B) Relative mPRα mRNA and protein expression for each fish was determined using quantitative PCR and western blot analyses, respectively.

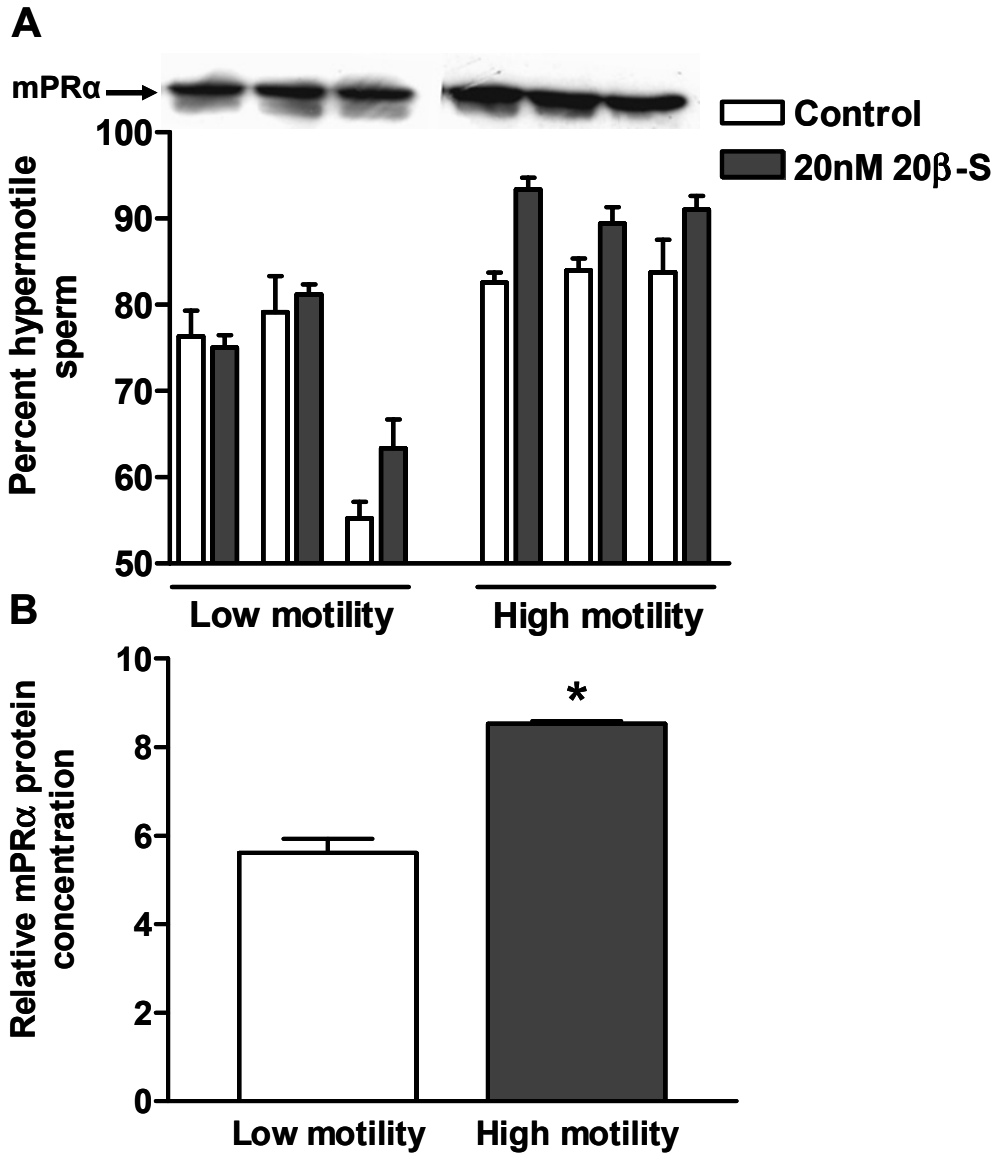

Figure 2.4. Concentration of mPRα protein in Atlantic croaker sperm and its association with sperm hypermotility. (A) Percent sperm hypermotility and mPRα protein expression in sperm membranes was determined for 6 individual donors. Representative mPRα western blots (gel loading=10 μg membrane protein) for each donor are displayed above bars showing percent hypermotile sperm after treatment with artificial seawater with 0.02% ethanol (control) or artificial seawater with 20 nM 20β-S. (B) Semi-quantitative densitometry of mPRα protein expression in croaker sperm membranes. Relative mPRα

concentrations were determined by measuring optical density of western blots shown in (A) using NIH ImageJ v. 1.26. Data represent means ± SEM. Statistically significant differences were determined using a Student's t-test ($p<0.05$; $n=3$).

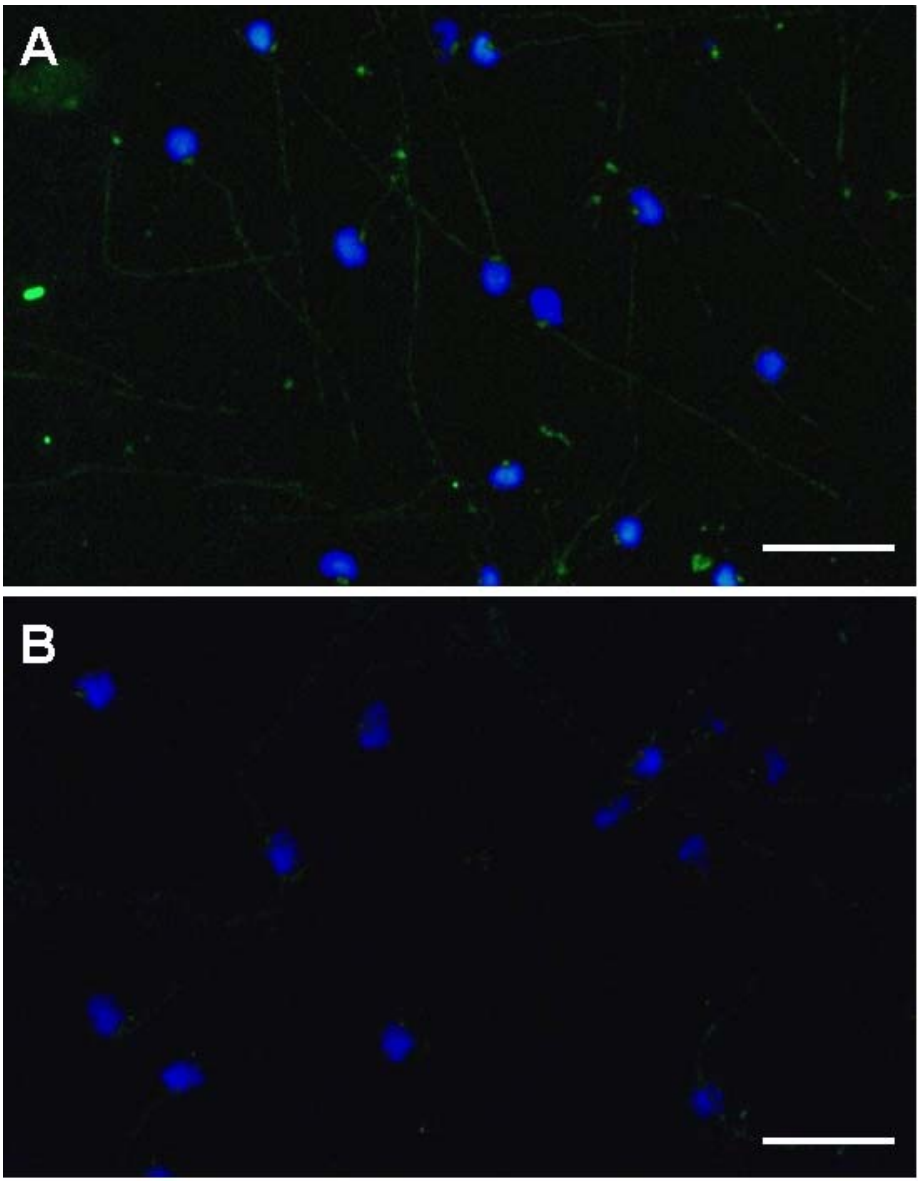

Figure 2.5. Immunocytochemical analysis of mPRα expression and its association with sperm hypermotility in Atlantic croaker. Expression of mPRα (green) in croaker sperm from a respresentative (A) high and (B) low motility donor. Sperm were fixed and incubated with a primary antibody directed against seatrout mPRα (1:1000) and an AlexaFluor 488 secondary antibody (1:2000). Sperm nuclei (blue) were counterstained with DAPI. Bar represents 10 μm. Results are typical of three experiments.

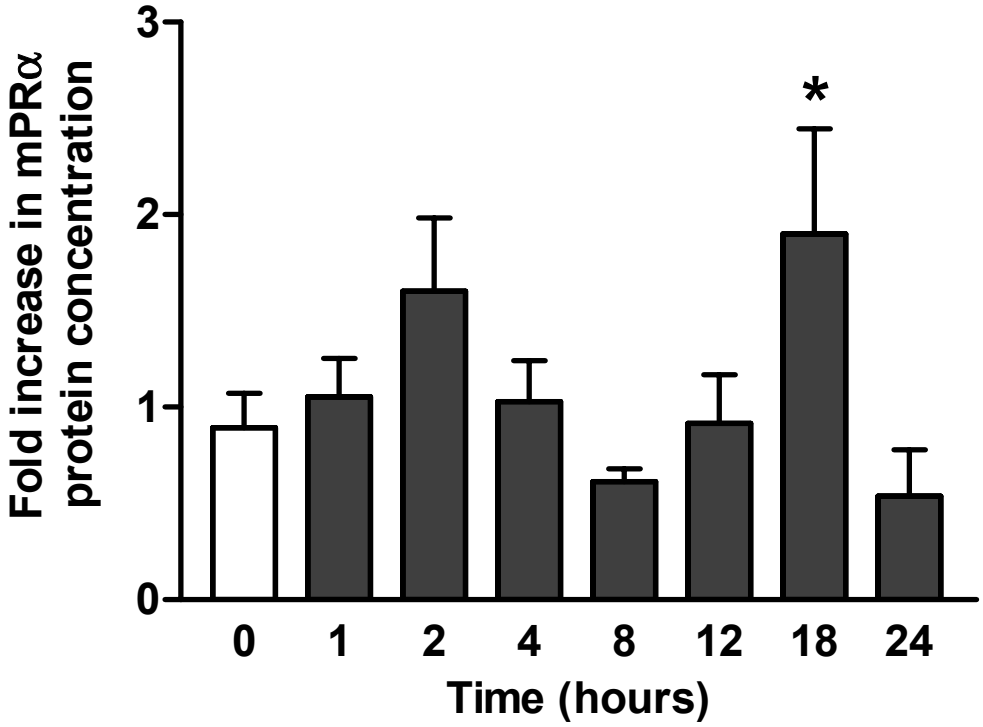

Figure 2.6. *In vitro* hormonal regulation of mPRα protein concentrations in the Atlantic croaker testis. Croaker testicular fragments were incubated in DMEM alone (control) or with 15 IU/ml human chorionic gonadotropin (hCG) for various time points. Data represent means ± SEM. Statistically significant differences were determined using a one-way ANOVA and Bonferonni's multiple comparison test (*$p<0.05$; n=4).

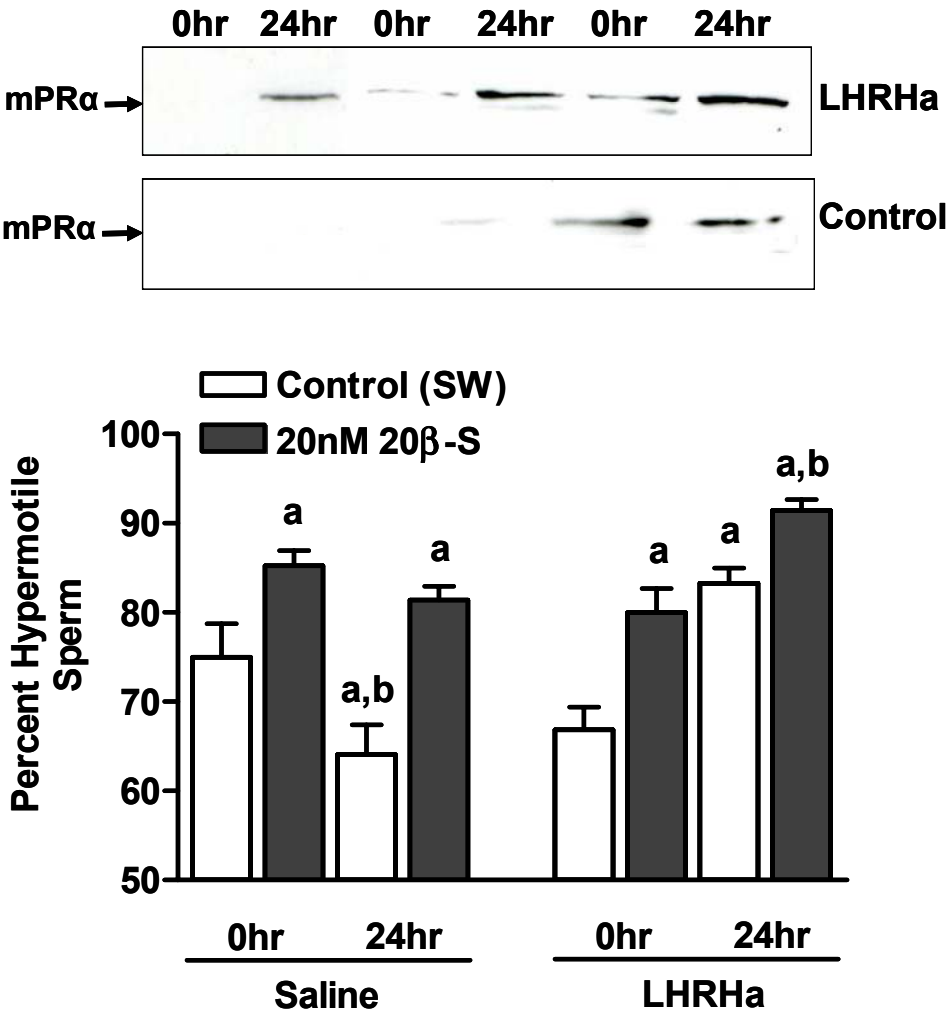

Figure 2.7. *In vivo* hormonal regulation of Atlantic croaker sperm hypermotility and mPRα protein concentrations. Sperm was collected from croaker before (0 hr) and after (24 hr) receiving an intraperitoneal injection of LHRHa (100 μg/kg body weight) or 0.8% saline (control) and analyzed for mPRα protein expression by western blot analyses and percent hypermotility following treatment with artificial seawater with 0.02% EtOH (vehicle control) or artificial seawater with 20nM 20β-S. Data represent means ± SEM. Statistically significant differences from a: 0 hr control or b: 0 hr 20 nM 20β-S for each treatment (*$p<0.05$) were determined using a one-way ANOVA and Dunnett's post-test (n=6).

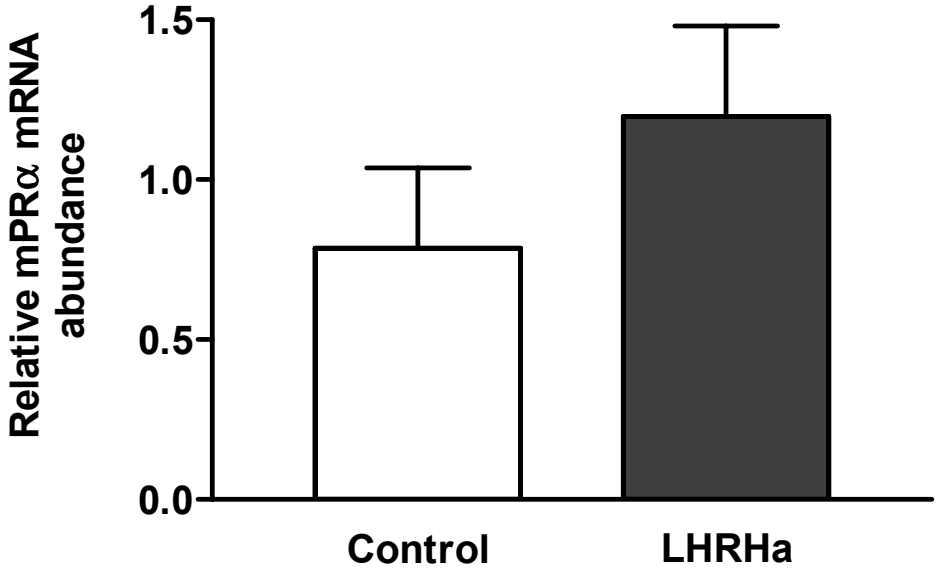

Figure 2.8. *In vivo* hormonal regulation of mPRα mRNA abundance in Atlantic croaker sperm. Croaker were given an intraperitoneal injection of 0.8% saline alone (control) or 100 μg/kg body weight luteinizing hormone releasing hormone analog (LHRHa) (n=3 groups for each treatment). Sperm were collected prior to and 24 hr after injection and analyzed for relative changes in mPRα mRNA abundance, normalized to croaker 18S rRNA gene by QPCR. Data represent means ± SEM and no statistical difference was found using a Student's *t*-test ($p>0.05$; n=5).

CHAPTER 3

A NOVEL PATHWAY INVOLVING mPRα, OLFACTORY G-PROTEINS AND MEMBRANE ADENYLYL CYCLASES INDUCES SPERM HYPERMOTILITY IN ATLANTIC CROAKER

Summary

Progestin stimulation of sperm hypermotility remains poorly understood despite having been described in numerous vertebrate species. In the present study, the hypothesis that progestins activate olfactory G-proteins (G_{olf}) and membrane adenylyl cyclases (mACs) to stimulate sperm hypermotility in the Atlantic croaker was tested. G_{olf} proteins were identified in croaker sperm membranes and activated following treatment with the progestin 17, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S). Treatment of sperm membranes with 20β-S caused an increase in cAMP production which was blocked by pre-treatment with cholera toxin and two mAC inhibitors; 2',5'-dideoxyadenosine (dd-Ado) and SQ22536. Moreover, pre-incubation of croaker sperm with both dd-Ado and SQ22536 resulted in a significant inhibition of 20β-S-stimulated sperm hypermotility. Finally, G_{olf} proteins and the novel membrane progestin receptor alpha (mPRα) were shown co-localize to the sperm midpiece and the flagella and co-immunoprecipitate from sperm membranes. Taken together, these results suggest that 20β-S activates mPRα which in turn activates G_{olf} proteins and mACs to stimulate hypermotility in Atlantic croaker sperm. Thus, these findings provide a plausible mechanism by which progestins

stimulate sperm hypermotility in croaker and provide the first evidence of hormonal activation of G_{olf} proteins in sperm of any species.

Introduction

Rapid actions of progestins to induce sperm hypermotility have been described in several mammalian species (Baldi et al., 1995; Uhler et al., 1992). However, the receptors and intracellular signaling pathways mediating this phenomenon are not currently known (Ho and Suarez, 2001; Luconi et al., 2004). In most of these species, progestins have also been shown to initiate the acrosome reaction, but it remains unclear whether the acrosome reaction and sperm hypermotility share a common progestin-mediated pathway (Baldi et al., 2002; Bedu-Addo et al., 2007; Marquez and Suarez, 2004). Progestin induction of sperm hypermotility has also been described in a teleost, the Atlantic croaker (*Micropogonias undulatus*) (Thomas et al., 2004). Treatment of croaker sperm with the endogenous progestin, 17, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S), results in a rapid increase in sperm hypermotility which is correlated with increases in intracellular cAMP and Ca^{2+} concentrations (Thomas, 2003; Thomas et al., 2004). These findings are consistent with observations in other vertebrate models (Harrison, 2003; Kobori et al., 2000; Parinaud and Milhet, 1996), but are not complicated by potential progestin activation of signaling pathways associated with the acrosome reaction, because croaker sperm lack an acrosome and do not undergo the acrosome reaction (Gwo, 1995). Thus, croaker is an excellent model for examining progestin stimulation of sperm hypermotility.

A progestin receptor has been biochemically characterized on croaker sperm membranes and is believed to be the receptor which mediates progestin-stimulated sperm hypermotility in this species (Thomas et al., 2005). Recent studies have identified the novel G-protein coupled receptor (GPCR)-like membrane progestin receptor alpha (mPRα) as a candidate for this receptor, as it is expressed in croaker sperm membranes and localized to the sperm midpiece (Thomas et al., 2005). Both recombinant and native mPRαs have been shown to bind progestins and activate inhibitory G-proteins to decrease cAMP concentrations in a variety of species (Hanna et al., 2006; Karteris et al., 2006; Thomas et al., 2007). Based on the observations that both cAMP and Ca^{2+} concentrations increase in croaker sperm in response to progestins, it is not clear which G-proteins might be activated by 20β-S. Candidates include the stimulatory family of G-proteins (G_s) which has both a long and a short form of G_s, as well as the olfactory-type G-protein (G_{olf}) (Jones and Reed, 1989). Unlike G_s, G_{olf} is expressed in high concentrations in the olfactory epithelium, but is also present in peripheral tissues such as the pancreas, testis and sperm (Baxendale and Fraser, 2003b; Jones and Reed, 1989; Regnauld et al., 2002; Spehr et al., 2004).

Although G_{olf} has only been cloned from a single non-mammalian species, *Xenopus* (Mezler et al., 2001), immunoreactive proteins have been demonstrated using a G_{olf} antibody in olfactory epithelia of three fish species (Frontini et al., 2003; Hansen et al., 2004; Hansen et al., 2003). G_{olf} expression has also recently been demonstrated in mammalian sperm, and therefore, it is possible that progestins could induce sperm hypermotility in croaker through activation of G_{olf}, resulting in production of cAMP via

membrane adenylyl cyclases (mACs) and subsequent opening of cAMP-gated Ca^{2+} channels. Although, mAC activation by GPCRs has been demonstrated in mammalian sperm (Baxendale and Fraser, 2003a; Spehr et al., 2004), the physiological role of mACs versus the ion-regulated soluble adenylyl cyclase (sAC) in cAMP production and sperm motility remains controversial (Jaiswal and Conti, 2003; Xie et al., 2006). The hypothesis that 20β-S stimulation of croaker sperm hypermotility requires activation of mACs and is associated with activation of G_{olf} proteins was tested in the present study. In addition, the potential involvement of the novel progestin receptor, mPRα, as the intermediary in progestin activation of G_{olf} proteins was examined.

Materials and Methods

Chemicals

17,20β,21-trihydroxy-4-pregnen-3-one and cortisol were purchased from Steraloids (Newport, RI). [^{35}S]GTPγS (~12000 Ci/mmol) was purchased from Amersham Pharmacia (Piscataway, NJ). An antibody against G_q was purchased from Biomol (Plymouth Meeting, PA) and the G_{olf} antibody from Santa Cruz Biotechnology (sc-383, Santa Cruz, CA). 2',5'-dideoxyadenosine and SQ22536 were purchased from Calbiochem (La Jolla, CA). All other chemicals and reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Animals

Atlantic croaker were captured by shrimp trawl and purchased from local bait shops. Fish were acclimated to the laboratory for 2 months before use in experiments in 12,000L recirculating tanks at 22-24ºC and a photoperiod of 11L:13D to stimulate gametogenesis. All procedures for this study were approved by the Institutional Animal Care and Use Committee of University of Texas at Austin.

Sperm collection and membrane isolation

Sperm were collected with a syringe from the cloaca of fully mature Atlantic croaker as described previously (Detweiler and Thomas, 1998). For all experiments, sperm was pooled from multiple (n=2-6) donors. Sperm membranes were isolated as described previously (Thomas et al., 1997) with modifications. Briefly, 3-5 ml of sperm was diluted in 10 ml of cold homogenization buffer (HAED; 25 mM HEPES, 10 mM NaCl, 10 mM $MgCl_2$, 1 mM dithioerythritol, 1 mM EDTA, pH 7.6) and centrifuged at 1000 x *g* to isolate sperm from seminal fluid. Sperm were resuspended in 10 ml HAED with HALT protease inhibitor cocktail (Pierce, Rockford, IL). Sperm suspensions were twice forced through a 23.5 gauge needle and sonicated at medium power for 6 sec on ice. Samples were then centrifuged at 500 x *g* for 20 min at 4ºC to remove nuclear material. The resulting supernatants were transferred to a clean tube and centrifuged at 17000 x *g* to obtain the membrane fraction. Isolated sperm membranes were used immediately or stored at -80ºC. Croaker tissues were collected from fish that were humanely sacrificed by cervical dislocation. Testicular, ovarian and olfactory epithelial

membranes were prepared in the same manner after 10 passes through a glass homogenizer.

Western blot analyses

Approximately 10 μg of membrane protein was added to loading buffer (0.5 M Tris-HCl, 10% SDS, 0.5% bromophenol blue, 10% glycerol) and resolved on 10% SDS-PAGE gels. After transfer to PVDF membranes, membranes were blocked in a solution containing 5% non-fat milk, 0.1% Tween 20 in phosphate buffered saline (PBS; 136 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 10 mM KH_2PO_4, pH 7.4). Membranes were rinsed in PBS and incubated overnight at 4ºC with antibodies directed against G_{i1-2}, G_q, or G_{olf} at a concentration of (1:500-1:1000). Since the G_{olf} antibody used could potentially cross-react with vertebrate G_s, western blots were performed on croaker sperm and testes with a G_s antibody to determine if two antibodies recognize similar proteins. This G_s antibody has previously been shown to recognize proteins in the ovary of a closely related species to croaker, the spotted seatrout (Pace and Thomas, 2005). Membranes were rinsed in PBS and incubated with a goat anti-rabbit horseradish peroxidase-linked secondary antibody (1:5000; Abcam, Cambridge, UK) in blocking solution. Proteins were then visualized using SuperSignal WestPico chemilumenescent substrate (Pierce, Rockford, IL). For G_{olf} western blots, the specificity of the reaction was confirmed by preabsorbing the antibody against the peptide antigen (Santa Cruz Biotechnology, Santa Cruz, CA).

Activation of G-proteins in croaker sperm membranes

Activation of G-proteins by hormonal treatment was assayed by measuring increased [^{35}S]GTPγS binding to croaker sperm membranes as described previously with modifications (Liu and Dillon, 2002; Pace and Thomas, 2005). Sperm membranes were suspended in binding buffer (50 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.6 mM EDTA, 0.1% BSA, pH 7.6) and pre-incubated with 10 μM GDP and 1 nM [^{35}S]GTPγS in the presence (non-specific binding) or absence (total binding) of 1 μM cold GTPγS. 250 μl aliquots of sperm membrane preparations were then added to tubes containing 100 nM steroid (20β-S or cortisol) or vehicle alone. Reactions were allowed to proceed for 20 min at room temperature with light shaking and were terminated by the addition of 750 μl of binding buffer containing 100 μM GDP/GTPγS (stop solution). 200μl of sperm membrane preparations were filtered through Whatman GF/B glass fiber filters using a vacuum manifold and washed with 25 ml of wash buffer (50 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.6 mM EDTA, pH 7.6). Total and non-specific [^{35}S]GTPγS binding was counted using a Beckman LS 6000SC scintillation counter (Fullerton, CA) and specific binding calculated by subtracting non-specific from total binding.

Identification of G-proteins activated in croaker sperm membranes

Immunoprecipitation of [^{35}S]GTPγS from croaker sperm membranes with specific G-protein alpha subunit antibodies was performed as described previously (Pace and

Thomas, 2005). Sperm membranes were pre-incubated with 4 nM [^{35}S]GTPγS in the presence (non-specific binding) or absence (total binding) of 4 μM cold GTPγS. Following incubation with 100 nM 20β-S or vehicle, stop solution was added and sperm membranes were centrifuged at 14000 x *g* for 15 min at 4ºC. Membrane pellets were resuspended in solubilization buffer (25 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, pH 7.6) with protease inhibitor cocktail and incubated for 1 hr at 4ºC with gentle shaking. Solubilized sperm membranes were then centrifuged at 14000 x g for 10 min and the supernatants were collected. Polyclonal antibodies directed against G_{i1-2}, G_q, G_s, G_{olf} or rabbit serum (negative control) were added to the supernatants at a concentration of 1:100 and incubated for 8 h at 4ºC. 50 μl of Protein A/G plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were then added and samples were incubated for 8 h at 4ºC. Beads were washed 3 times with wash buffer (50 mM Hepes, 100 mM NaCl, 50 mM Na_2HPO_4, 100 μM NaF, 1% Triton X-100, 0.1% SDS pH 7.6) and boiled in 0.5% SDS for 10 min. Samples were centrifuged, the supernatants collected and specific [^{35}S]GTPγS binding determined.

Measurement of cAMP production by croaker sperm membranes

The [^{35}S]GTPγS immunoprecipitation experiments showed that G_{olf} proteins were activated in croaker sperm membranes by 20β-S, suggesting the 20β-S-induced increase in cAMP is through activation of G_{olf} proteins and mACs. Therefore, pre-treatment of sperm membranes with the G_{olf} activator, cholera toxin (CTX) (Jones et al., 1990), should decrease cAMP production by 20β-S, because CTX would deplete the pool of

unactivated G_{olf} proteins coupled to receptors. Moreover, the increase in cAMP should be blocked by the specific mAC inhibitors; 2',5'-dideoxyadenosine (dd-Ado) and SQ22536 and stimulated by the mAC activator, forskolin. To test this, sperm membranes were resuspended in buffer (75 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM EDTA, pH 7.6) to a total protein concentration of ~1 mg/ml. Membrane suspensions were pre-incubated with inhibitors (12.5 μg/ml CTX or iCTX; 50 or 100μM dd-Ado; 0.5mM SQ22536) or vehicle (0.01% DMSO) for 20 min at room temperature and then incubated in an equal volume of assay buffer (0.2 mM ATP, 10 nM GTP, 0.50 mM phosphoenolpyruvate, 20 μg pyruvate kinase, 2 mM IBMX) with 20β-S (20 nM final concentration) or vehicle (0.01% ethanol) for 1 min. CTX was activated by incubation at 37ºC for 30 min or heat-inactivated by boiling for 30 min prior to addition to sperm membranes. For forskolin treatments, assay buffer containing forskolin (10 μM final concentration) was added to sperm membranes for 5 min after the 20 minute pre-incubation with 100 μM dd-Ado or vehicle. Samples were then boiled for 5 min and centrifuged at 14000 x *g* for 10 min. Supernatants were collected, diluted 10 to 20-fold and cAMP concentrations were determined using a commercial cAMP EIA kit following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). For all experiments, data were normalized to control samples due to the variation of cAMP produced between samples, which is a noted problem in evaluating cAMP concentrations of numerous vertebrate sperm donors or donor groups (Harrison, 2003).

Sperm motility analyses

Sperm were collected and sperm motility experiments were performed as described previously (Thomas and Doughty, 2004). Sperm were diluted 100-fold and pre-incubated with physiological saline with mAC inhibitors; dd-Ado (50 or 100 μM) or SQ22536 (0.5 mM) for 20 min at room temperature. Control samples were incubated with vehicle alone (DMSO; 0.1%). Sperm were then treated with physiological saline containing 20β-S (20 nM final concentration) or vehicle (ethanol, 0.02%) for 1 min or with forskolin (10 μM final concentration) for 5 min. A 2 μl aliquot of each sperm suspension was then placed on a microscope slide and diluted with 25 μl of artificial seawater. A coverslip was placed on the slide and sperm were viewed using dark field microscopy. Each experiment was recorded using a CCD camera (Cohu Electronics, San Diego, CA) and a VHS recorder and the percent of sperm displaying a rapid velocity, an increased rate of turning and rapid flagellar beating characteristic of hypermotility, for each treatment was determined.

Immunocytochemistry

Sperm were collected and diluted 1000-fold in cold PBS. Aliquots of sperm suspensions were spread on poly-L-lysine coated slides and air dried for 30 min. Sperm were fixed in 4% paraformaldehyde for 20 min on ice and rinsed with PBS. Slides were then blocked in 2% bovine serum albumin (BSA) for 2 h. For immunocytochemical localization of G_{olf} proteins, 0.3% Triton X-100 was added to the blocking buffer to permeablize sperm. After 3 rinses with PBS, slides were incubated with a rabbit anti-G_{olf}

antibody (1:500) or a rabbit anti-mPRα antibody (1:1000) overnight at 4ºC. As a negative control, antibodies were pre-absorbed against the peptide antigen. Slides were rinsed with PBS and incubated for 2 h at room temperature with an AlexaFluor 488 goat anti-rabbit secondary antibody (1:1000; Molecular Probes, Eugene, OR). To visualize sperm nuclei, slides were treated with 300 nM 4′,6-diamidino-2-phenylindole (DAPI) for 10 min, rinsed with PBS and coverslips were mounted using ProLong Gold Antifade Reagent (Molecular Probes, Eugene, OR). Sperm were visualized using a Nikon Eclipse E600 fluorescent microscope.

Co-immunoprecipitation of G_{olf} proteins and mPRα

Sperm membrane pellets were solubilized by resuspension in HAED buffer with 1% Triton X-100 at a final protein concentration of ~1 mg/ml. Solubilized membrane proteins were then immunoprecipitated with an anti-G_{olf} antibody or normal rabbit IgG (negative control) using a Seize Immunoprecipitation Kit (Pierce, Rockford, IL) following the manufacturer's instructions. After elution from the column, immunoprecipitated proteins were run on a 10% SDS polyacrylamide gel and western blot analyses were performed as described above using an antibody against mPRα (Thomas et al., 2007). A freshly prepared croaker sperm membrane sample was used as a positive control.

Statistical Analyses

For all experiments, data are presented as means ± SEM. Statistical significance was determined using a one-way ANOVA and Dunnett's multiple comparison post-test or Student's *t*-test using GraphPad Prism Software (San Diego, CA).

Results

Expression of G_{olf} proteins in croaker sperm

Western blot analyses demonstrated the presence of G_{i1-2}, G_q, G_s, and G_{olf} proteins in sperm and testicular membranes (Fig. 3.1A). Bands of approximately 45 kDa were detected in croaker sperm and testicular membranes with the G_{olf} protein antibody, which corresponds to the predicted size of this protein, as well as the reported size of this protein in other fish species using the same antibody (Hansen et al., 2004; Hansen et al., 2003). In both sperm and testes, the G_s antibody used recognized two proteins, neither of which were the same size as the protein detected using the G_{olf} antibody. To further confirm that the G_{olf} antibody did not cross-react with G_s, western blot analyses were performed on croaker olfactory epithelium and ovary which should express high concentrations of G_{olf} and G_s, respectively. These experiments showed that the G_{olf} antibody recognized a 45 kDa protein in olfactory epithelium, but no proteins were detected in the ovary (Fig. 3.1B).

Activation of G-proteins by 20β-S in croaker sperm membranes

Possible activation of G-proteins in croaker sperm by 20β-S was assessed by measuring binding of the non-hydrolyzable GTP analog, [^{35}S]GTPγS, to sperm membranes. Binding of [^{35}S]GTPγS to sperm membranes significantly increased following treatment with 100 nM 20β-S (Fig. 3.2A). Treatment of sperm membranes with another C21 steroid, cortisol, did not activate G-proteins.

The identity of the G-protein activated by 20β-S in croaker sperm membranes was determined by immunoprecipitating the [^{35}S]GTPγS from sperm membranes with specific antibodies directed against different G-protein α subunits. Treatment with 100 nM 20β-S resulted in G_{olf} protein activation as shown by a significant increase in the amount of [^{35}S]GTPγS immunoprecipitated with the G_{olf} protein antibody compared to controls (Fig. 3.2B). The G_s antibody, did not immunoprecipitate any [^{35}S]GTPγS . The amounts of [^{35}S]GTPγS immunoprecipitated with pre-immune rabbit serum and the G_{i1-2}, G_q protein antibodies were unaltered by 20β-S treatment.

Inhibition of 20β-S-stimulated adenylyl cyclase activity in croaker sperm membranes by cholera toxin

Treatment of sperm membranes with 20 nM 20β-S caused a significant increase in cAMP production compared to vehicle controls (Fig. 3.3). Pre-treatment with 12.5 μg/ml CTX prevented the 20β-S-induced increase in cAMP (Fig. 3.3). In contrast, pre-treatment with heat-inactivated CTX (iCTX) was ineffective in blocking 20β-S-stimulation of AC activity in sperm membranes. Pre-treatment of sperm membranes with 12.5 μg/ml CTX

or iCTX alone did not result in a significant change in AC activity. To verify that CTX can activate croaker stimulatory G- proteins, sperm membranes were treated with CTX in the presence of reagents necessary for cAMP synthesis (0.1 mM ATP, 5 nM GTP, 0.25 mM phosphoenolpyruvate, 10 μg/100 μL pyruvate kinase, 1 mM IBMX) for 5 min, which resulted in a significant increase in AC activity (Fig. 3.3).

Activation of membrane adenylyl cyclases by 20β-S in croaker sperm membranes

To determine if mACs are activated by 20β-S, the effects of mAC inhibitors on AC activity were examined. Pre-treatment of sperm membranes with 50 or 100 μM 2',5'-dideoxyadenosine (dd-Ado) significantly decreased cAMP production in response to 20 nM 20β-S (Fig. 3.4A). Production of cAMP following treatment with the specific mAC activator forskolin was used to confirm the presence of mAC(s) in croaker sperm membranes. Treatment of sperm membranes with 10 μM forskolin resulted in an increase in cAMP synthesis, which was blocked by pre-incubation of sperm membranes with 100 μM dd-Ado (Fig. 3.4A). A second mAC inhibitor, SQ22536 (0.5 mM) was also effective in inhibiting mAC activity in response to 20β-S in sperm membranes (Fig. 3.5A).

Stimulation of croaker sperm hypermotility by 20β-S activation of membrane adenylyl cyclases

To verify that activation of mACs mediates 20β-S-induced croaker sperm hypermotility, the ability of two cell-permeable mAC inhibitors to block this action of 20β-S was investigated in an *in vitro* sperm motility bioassay. Pre-treatment of live sperm

with 50 or 100 μM dd-Ado and 0.5 mM SQ22536 were all effective in blocking induction of sperm hypermotility by 20 nM 20β-S (Fig. 3.4B and 3.5B). Similar to the effects of treatment with 20β-S, incubation of sperm with 10 μM forskolin resulted in an increase in the percentage of hypermotile sperm which was blocked by pretreatment of sperm with 100 μM dd-Ado (Fig. 3.4B).

Co-localization and association of G_{olf} proteins and mPRα in croaker sperm

Immunocytochemcial studies show that G_{olf} proteins are expressed in the sperm midpiece and flagella (Fig. 3.6A). The specificity of the immunoreactions was confirmed by pre-absorbing the antibody against peptide antigens (data not shown). Expression of mPRα is also localized primarily to the sperm midpiece, with weaker expression occurring on the flagella (Fig. 3.6B). Furthermore, co-immunoprecipitation experiments were performed to determine if mPRα is coupled to G_{olf} proteins. Incubation of solubilized sperm membranes in the presence of anti-G_{olf} antibody resulted in the immunoprecipitation of an 80 kDa protein which corresponds to the size of the major mPRα protein detected in croaker sperm membranes (Thomas et al., 2005) (Fig. 3.6C). Protein bands of this size are commonly observed with the mPR family of receptors and are thought to represent a mPRα dimer (Thomas et al., 2007). As a negative control sperm membranes were incubated with normal rabbit IgG, which did not immunoprecipitate mPRα.

Discussion

Here we demonstrate that activation of mACs is necessary for progestin induction of hypermotility in Atlantic croaker sperm and involves activation of G_{olf} proteins. To our knowledge, the results of this study provide the first evidence that hormones can induce responses in target cells through activation of G_{olf} proteins. The finding that AC activity in sperm membranes was stimulated by 20β-S and that this effect was blocked by pre-treatment with CTX and two mAC inhibitors, dd-Ado and SQ22536, indicates the signaling pathway induced by the progestin hormone involves activation of a stimulatory G-protein and mACs. Moreover, activation of mACs is critical for hormonal induction of sperm hypermotility since pre-incubation of croaker sperm with both dd-Ado and SQ22536 effectively blocked this stimulatory effect of 20β-S. Stimulation of mAC activity was associated with activation of G_{olf} proteins by 20β-S and no evidence was obtained for activation of G_s proteins in sperm membranes by this progestin. Both G_{olf} proteins and mPRα were shown to be localized to the midpiece of croaker sperm, which is consistent with their hypothesized roles as intermediaries in 20β-S-induced sperm hypermotility. Finally, mPRα was shown to co-immunoprecipitate with G_{olf} proteins from sperm membranes, demonstrating that these two proteins physically associate with one another. Taken together, these findings suggest that 20β-S activates a G_{olf}/mAC pathway via mPRα to induce hypermotility in Atlantic croaker sperm (Fig. 3.7). They also provide the first indication that some progestin hormone actions in vertebrate sperm are mediated through activation of G-proteins.

Several lines of evidence suggest that the G-protein activated by 20β-S and detected by the G_{olf} antibody is G_{olf}, and not G_s, which share 88% amino acid sequence homology in mammals (Jones and Reed, 1989). The G_{olf} antibody recognizes a 45 kDa protein on western blots of olfactory epithelia from croaker and three other fish species; the sea lamprey, goldfish and channel catfish (Frontini et al., 2003; Hansen et al., 2004; Hansen et al., 2003). A similar 45 kDa protein was also detected in croaker sperm and testes, which corresponds to the size of the human G_{olf} (Zigman et al., 1993). In contrast, the G_{olf} antibody did not cross-react with any of the protein bands on western blots of croaker sperm and testicular tissue detected with the G_s antibody. Similarly, the G_{olf} antibody failed to recognize any protein on western blots of croaker ovarian tissue, which expresses G_s in croaker and a closely related species, the spotted seatrout (Pace and Thomas, 2005). Finally, the immunoprecipitation experiments showing that G_{olf} proteins, but not G_s proteins, were activated by 20β-S provide further evidence for the specificity of the G_{olf} antibody and the identity of the G-protein activated by 20β-S. Thus, we conclude that G_{olf} proteins, and not G_s proteins, are activated by 20β-S in croaker sperm membranes.

The teleost progestin hormone, 20β-S, has previously been shown to act directly on sperm from Atlantic croaker and several other marine fishes in a concentration dependent manner to induce hypermotility *in vitro* (Thomas, 2003; Thomas et al., 2006; Thomas et al., 2004). 20β-S is thought to act through specific receptors that have been characterized biochemically on croaker and spotted seatrout sperm membranes (Thomas et al., 1997; Thomas et al., 2005), resulting in rapid increases in intracellular cAMP and

Ca^{2+} concentrations (Thomas, 2003). Thus, the basic mechanism of progestin induction of sperm hypermotility in these teleosts is similar to that in humans and other mammals (Harrison, 2003; Kobori et al., 2000; Parinaud and Milhet, 1996). A major advantage of teleost sperm models is that the signaling pathways involved in progestin-induced hypermotility can be studied in isolation, without the complication of progestin pathways associated with the acrosome reaction, because teleost sperm does not undergo this process (Gwo, 1995). Hormonal treatment of teleost sperm *in vitro* also permits direct progestin effects on sperm motility to be distinguished from indirect genomic actions involving alterations in seminal fluid composition, which has been demonstrated in several fish species (Miura et al., 1991; Miura et al., 1992).

Our findings on the presence and localization of G_{olf} in croaker sperm are in agreement with previous studies in mammals. G_{olf} proteins have been found in human, rat and mouse male germ cells and as shown with croaker sperm, are expressed in the midpiece and flagella of human and mouse sperm (Baxendale and Fraser, 2003b; Defer et al., 1998; Spehr et al., 2004). The presence of various mACs has also been demonstrated in human, rat and mouse male germ cells and in particular, mACIII has been shown to co-localize with G_{olf} proteins in these species (Baxendale and Fraser, 2003a; Defer et al., 1998; Spehr et al., 2004). Indirect evidence for the presence of mACs in fish sperm was obtained in the present study using a specific mAC activator, forskolin, and inhibitor, dd-Ado. Thus, the presence of both G_{olf} and mACs in sperm appears to be conserved in vertebrates. However, the precise roles of G_{olf} activation of mACs in vertebrate sperm physiology remain to be clarified.

The role of mACs versus soluble adenylyl cyclases (sACs) in the regulation of sperm hypermotility via cAMP synthesis remains controversial. Studies have clearly demonstrated that sAC is present in mammalian sperm and is regulated by Ca^{2+} and HCO_3^- to increase cAMP production (Jaiswal and Conti, 2003), and that sAC is essential for sperm motility in mice (Esposito et al., 2004; Hess et al., 2005; Xie et al., 2006). However, the presence and activation of mACs have also been demonstrated in mammalian germ cells and mature sperm (Baxendale and Fraser, 2003a; Defer et al., 1998; Gautier-Courteille et al., 1998; Spehr et al., 2004). Interestingly, disruption of mACIII, which is the primary target of activated G_{olf} proteins, results in impaired sperm function in mice, including decreases in both sperm motility and fertilization success (Livera et al., 2005). In the present study, treatment of sperm membranes with 20 nM 20β-S and forskolin resulted in an increase in cAMP production, which was inhibited by pre-treatment with the mAC inhibitor dd-Ado, suggesting that mACs are present in croaker sperm. Furthermore, dd-Ado and another mAC inhibitor, SQ22536, blocked 20β-S-stimulated sperm hypermotility, demonstrating that it is mediated through 20β-S activation of mACs, which in turn are likely activated by G_{olf} proteins. However, the primary stimulus for initiation of sperm motility in croaker is exposure to a hyperosmotic medium upon their release into seawater, which in the present study (control groups) resulted in approximately 70% motile sperm. This requires the opening of voltage-sensitive ion channels and the presence of extracellular Ca^{2+} and HCO_3^- (Detweiler and Thomas, 1998). These findings suggest that croaker, like mammals, may require sAC activation as a primary regulator of sperm function, although the presence of sACs in

sperm has not been described for any teleost species. However, our results indicate that activation of G_{olf} and mACs by 20β-S also influences sperm motility by inducing hypermotility.

The observation that mPRα and G_{olf} are co-localized and co-immunoprecipitate suggests that mPRα mediates progestin-stimulated sperm hypermotility in croaker through activation of G_{olf} proteins. This is the first evidence of a direct association of a progestin receptor with any G-protein in the sperm of a vertebrate species. Other putative progestin receptors have been suggested to mediate rapid actions of progestins in sperm, including the nuclear progesterone receptor B isoform in humans (Gadkar et al., 2002; Shah et al., 2005) as well as a putative novel membrane-bound progestin receptor originally cloned from porcine liver (Buddhikot et al., 1999). Both of these receptor proteins are localized to the posterior head and acrosomal region of mammalian sperm, suggesting that they are involved in progestin stimulation of acrosome reaction. In contrast, mPRα has been shown to localize to the midpiece of croaker sperm (Thomas et al., 2005), which was confirmed in the present study. Furthermore, preliminary evidence from our laboratory has shown that human mPRα is also localized to the midpiece, and not the acrosome of human sperm (Thomas and Tubbs, unpublished obs). Taken together, these findings suggest that mPRα is involved in progestin stimulation of sperm hypermotility, and not the acrosome reaction, as has been proposed for other putative progestin receptors in sperm.

The physiological relevance of progestin stimulation of sperm hypermotility remains a controversial issue. One hypothesis is that progesterone released in follicular

fluid following ovulation serves as a chemoattractant to stimulate sperm chemotaxis (Jaiswal et al., 1999; Teves et al., 2006). Recently, activation of G_{olf} and mACs in human sperm through the odorant receptor hOR-17 has been suggested to be a signaling pathway which mediates sperm chemotaxis (Spehr et al., 2004). The floral odorant burgeonal and a variety of other floral compounds activate this pathway in human and rodent sperm (Fukuda et al., 2004; Spehr et al., 2004; Spehr et al., 2006). However, they are not present in the human oviduct and the endogenous ligands stimulating the hOR-17/G_{olf}/mAC pathway are unknown (Spehr et al., 2006). In light of the present study, it is possible that progestins mediate croaker sperm chemotaxis by activating G_{olf} and mACs through mPRα. Croaker sperm move in a circular trajectory and hypermotility is correlated with a rapid increase in velocity and decrease in turning radius. As a result, hypermotile croaker sperm remain in the area in which hyperactivation occurs. Croaker oocytes are fertilized externally and approximately $5x10^5$ oocytes are ovulated during spawning. Since ovarian production of 20β-S increases dramatically prior to ovulation (Patino and Thomas, 1990) it is possible that sufficient 20β-S concentrations are released with ovarian fluid to simulate sperm hypermotility as sperm and eggs are released simultaneously in the water during spawning. However, this hypothesis and a possible chemotaxic action of progestins through the mPRα/G_{olf}/mAC pathway on croaker sperm remain to be tested.

Perhaps one of the most significant findings of the present study was that steroid receptors directly couple to, and activate G_{olf} proteins at low physiological concentrations, to elicit a biological response. This finding further expands the repertoire of signal transduction pathways through which steroid hormones can act and may explain

some of the pleiotropic actions of steroids, particularly their intraspecific actions as pheromones. It is noteworthy that urinary metabolites of 20β-S and other progestins secreted by a variety of female fish species are detected in the olfactory epithelia of conspecific males and act as pheromones, inducing both behavioral and hormonal responses (Kobayashi et al., 2002; Sorensen et al., 2004). Activation of G_{olf} proteins has only been reported previously in the neuroepithelium (Jones and Reed, 1989) and sperm (Spehr et al., 2004) in response to odorants, and in the central nervous system in response to the neurotransmitter dopamine (Zhuang et al., 2000). The present results indicate that G_{olf} proteins have a more widespread role in transducing extracellular chemical signals into intracellular ones, and are also activated by steroid hormones. The functions of other tissues that express G_{olf} proteins, such as the pancreatic islets (Regnauld et al., 2002), are also influenced by steroid hormones. Therefore, steroids could act in these tissues via a similar mechanism to that identified in croaker sperm, through activation of G_{olf} proteins.

In conclusion, we suggest that 20β-S activates G_{olf} proteins to stimulate sperm hypermotility in Atlantic croaker. This mechanism involves increases in cAMP production through downstream activation of mACs. An association between mPRα and G_{olf} proteins indicates that mPRα mediates 20β-S-stimulated sperm hypermotility. This is the first such evidence of the association of a progestin receptor with a specific signaling pathway believed to regulate sperm hypermotility. These findings also present the first evidence of progestin activation of G_{olf} proteins in sperm or other tissues of any species.

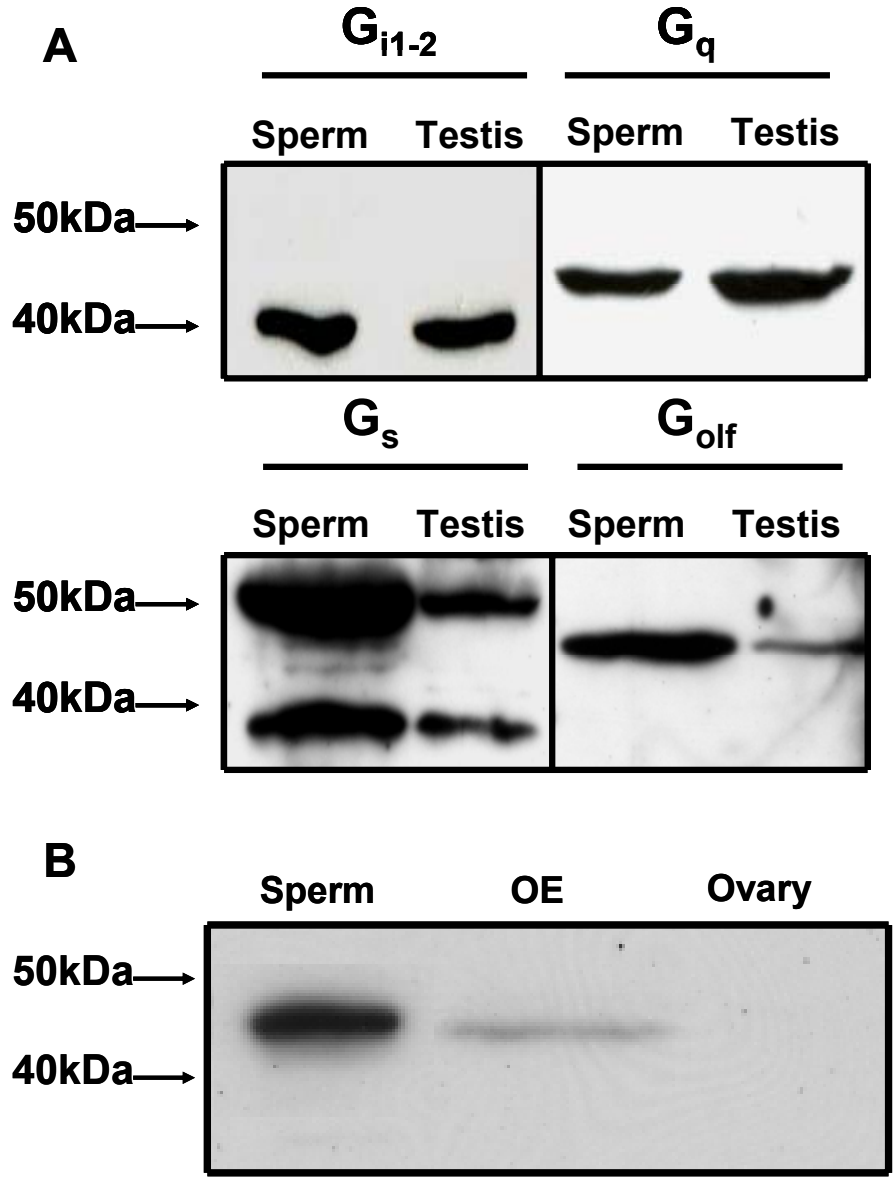

Figure 3.1. Identification of G-proteins in Atlantic croaker sperm membranes. (A) Representative western blot analyses of G-proteins in membrane preparations of croaker sperm and testes using antibodies directed against G_{i1-2}, G_q, G_s and G_{olf} (gel loading=10 μg). (B) Western blot analyses of membrane preparations of croaker sperm, olfactory epithelium (OE) and ovary using and anti-$G_{olf/s}$ antibody.

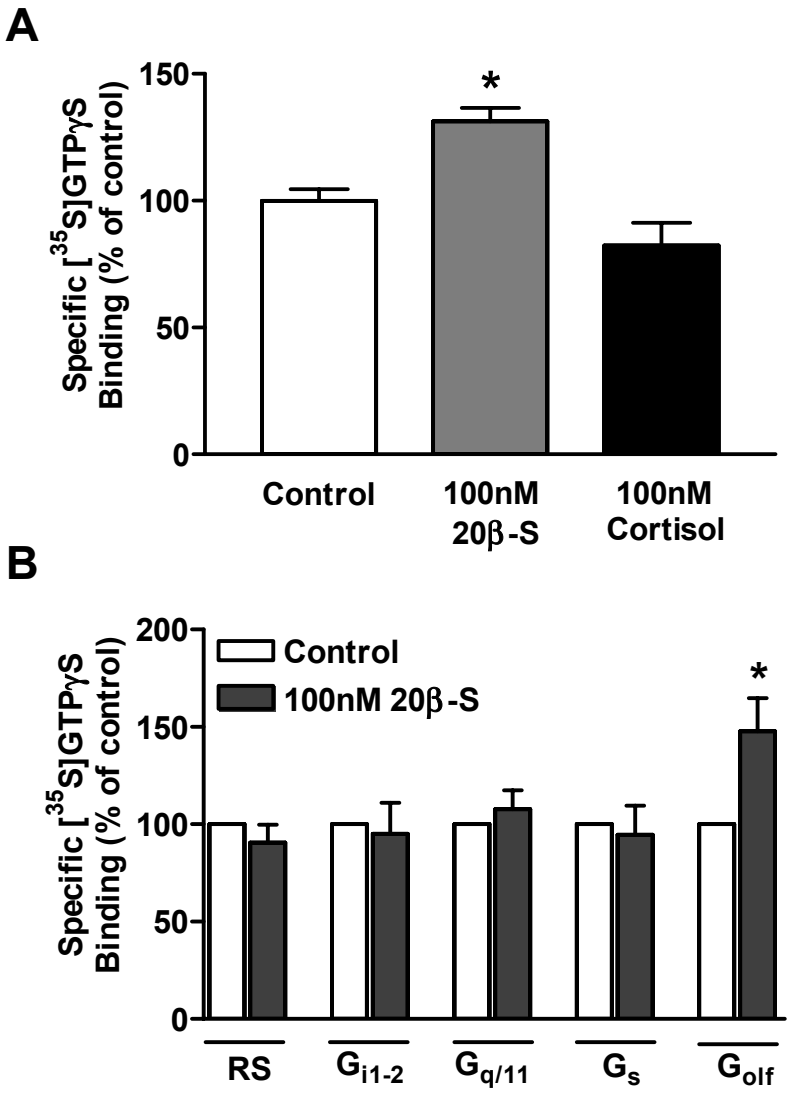

Figure 3.2. G-protein activation by 20β-S in Atlantic croaker sperm membranes. (A) Croaker sperm membranes were pre-incubated for 20 min with or without 12.5 μg/mL cholera toxin (CTX) and 1 nM [^{35}S]GTPγS in the presence (non-specific binding) or absence (total binding) of 1 μM cold GTPγS. Samples were then treated with 100 nM 20β-S, 100 nM cortisol or 0.01% EtOH (control) for 20 min and specific binding of [^{35}S]GTPγS was determined. (B) Croaker sperm membranes, treated as described above with [^{35}S]GTPγS in the presence or absence of GTPγS and 20β-S, were solubilized and

incubated with antibodies directed against G_{i1-2}, G_q, G_s, G_{olf} proteins or rabbit serum as a negative control. G-proteins were immunoprecipitated and activation of different G-proteins was determined from a comparison of the amount of specific [^{35}S]GTPγS binding in the 20β-S treatment group relative to that in the EtOH control group. Bars represent mean ± SEM percent of specific [^{35}S]GTPγ-S binding compared to untreated controls. Statistically significant differences from control samples (*$p<0.05$) were determined using a one-way ANOVA and Dunnett's post-test for [^{35}S]GTPγS binding experiments (n=5) and Student's *t*-test for immunoprecipitation studies for each G-protein antibody tested (n=4) prior to normalization of data to control values.

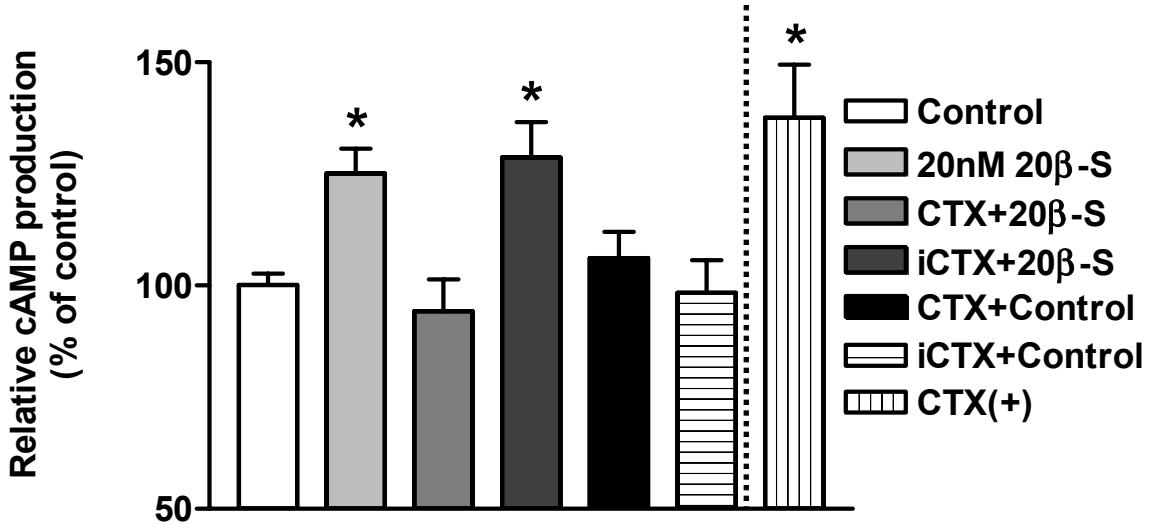

Figure 3.3. CTX inhibition of 20β-S-stimulated cAMP production by Atlantic croaker sperm membranes. Production of cAMP was measured following 1 min treatment with 20nM 20β-S after pre-incubation with 12.5 μg/mL CTX or iCTX. (B) To confirm activation of G-proteins by cholera toxin, sperm membranes were incubated with CTX in the presence of 0.1 mM ATP, 5 nM GTP, 0.25 mM phosphoenolpyruvate, 10 μg pyruvate kinase, 1 mM IBMX for 5 min (CTX(+)). Data represent means ± SEM. Statistically significant differences from control samples (*$p<0.05$) was determined using a one-way ANOVA and Dunnett's post-test (n=5).

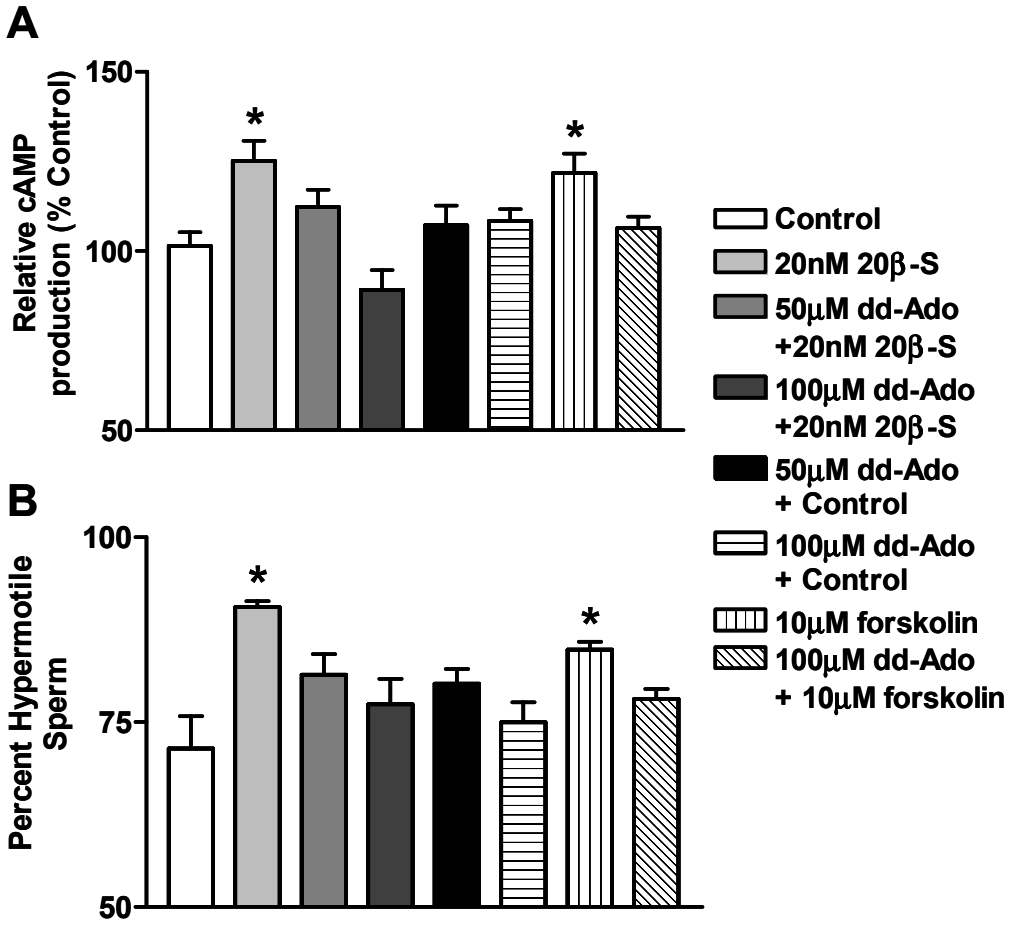

Figure 3.4. Effect of dd-Ado on 20β-S-stimulated cAMP production and hypermotility in Atlantic croaker sperm. (A) Production of cAMP was measured following treatment with 20nM 20β-S for 1 min after a 20 min pre-incubation with 50 or 100 μM 2',5'-dideoxyadenosine (dd-Ado). (B) Percent hypermotile sperm was determined following 1 min treatment with 20 nM 20β-S after a 20 min pre-incubation with 50 or 100 μM dd-Ado. For both experiments a group of (A) sperm membranes or (B) live sperm were incubated for 5 min with 10 μM of the mAC activator, forskolin. Data represent means ± SEM. Statistically significant differences from controls (*$p<0.05$ for cAMP, *$p<0.001$ for sperm motility) were determined using a one-way ANOVA and Dunnett's post-test (n=5).

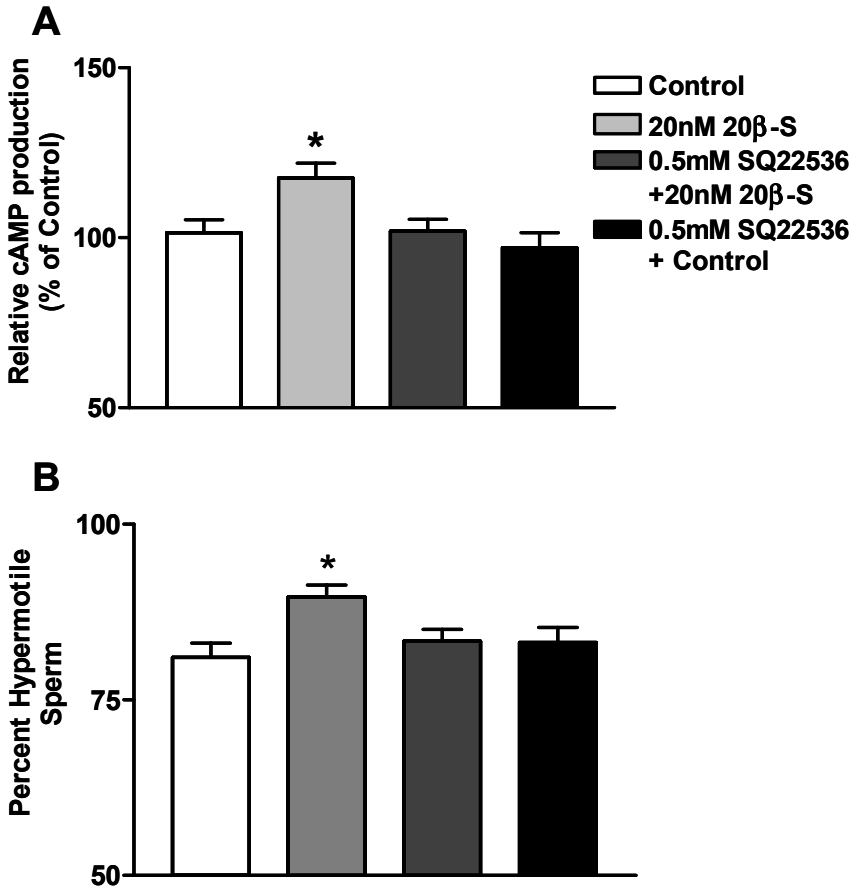

Figure 3.5. Effect of SQ22536 on 20β-S-stimulated cAMP production and hypermotility in Atlantic croaker sperm. (A) Production of cAMP was measured following treatment with 20 nM 20β-S for 1 min after incubation after 20 min pre-incubation with 0.5 mM SQ22536. (B) Percent hypermotile sperm was determined following 1 min treatment with 20 nM 20β-S after a 20 min pre-incubation 0.5 mM SQ22536. Data represent means ± SEM. Statistically significant differences from control samples (*$p<0.05$ for cAMP, *$p<0.001$ for sperm motility) was determined using a one-way ANOVA and Dunnett's post-test (n=5).

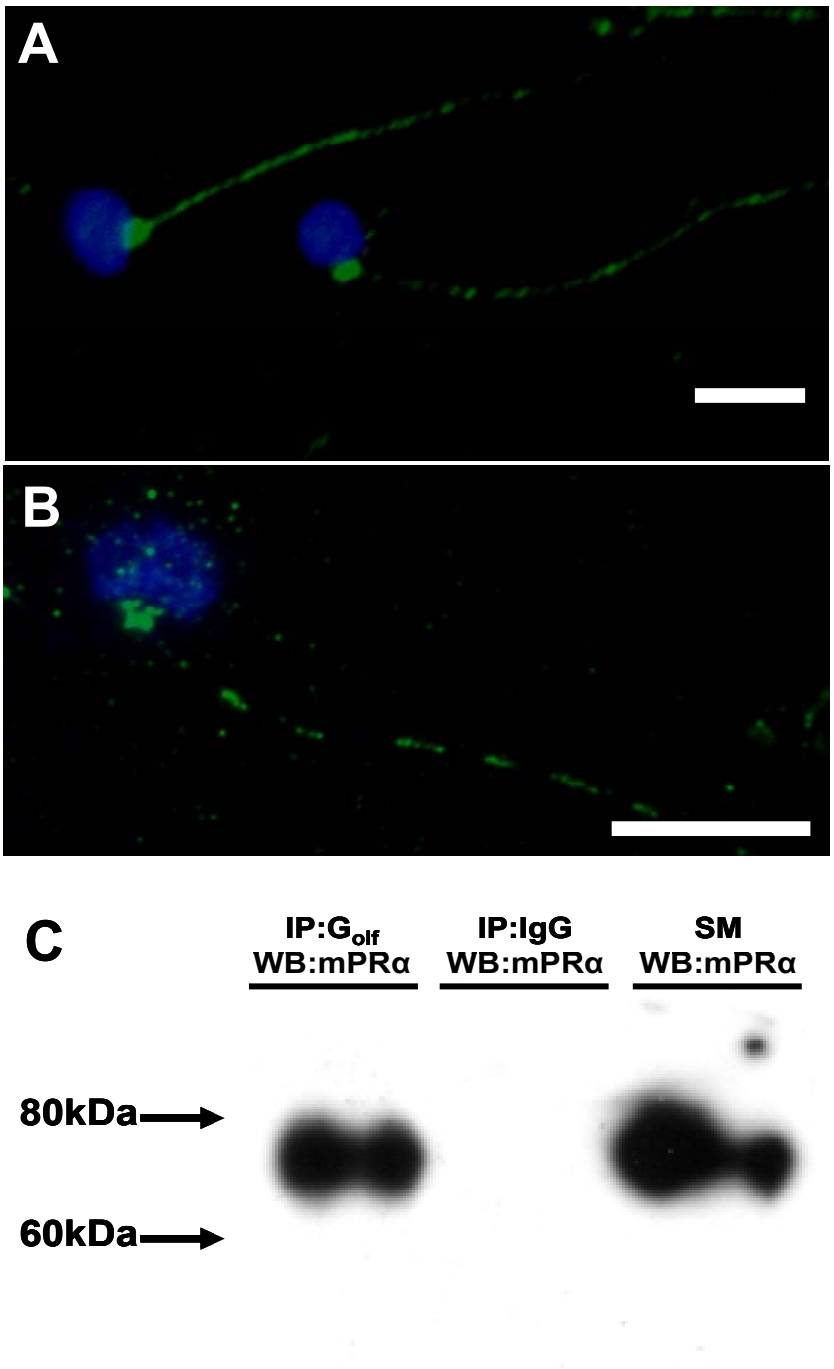

Figure 3.6. Co-localization and association of G_{olf} proteins with mPRα in Atlantic croaker sperm. (A) Immunocytochemical localization of G_{olf} and (B) mPRα (both in green) in croaker sperm using anti-G_{olf} and anti-mPRα antibodies, respectively. Nuclei (blue) were counterstained with DAPI. Bar represents 5 μm. Both experiments were

repeated 3 times. (C) Co-immunopreciptation of G_{olf} and mPRα from solubilized sperm membranes incubated with rabbit anti-G_{olf} antibody (IP:G_{olf}) immobilized on a commercial column. Immunoprecipitated proteins were eluted from the column, and 3 μg of total protein run on a 10% SDS-PAGE gel and analyzed by western blot analyses using an antibody directed against mPRα (WB:mPRα). As a negative control, solubilized sperm membranes were incubated in a column with normal rabbit IgG (IP:IgG). As a positive control, freshly prepared sperm membranes (SM) were run on the same gel (SM). Data are typical of three independent experiments.

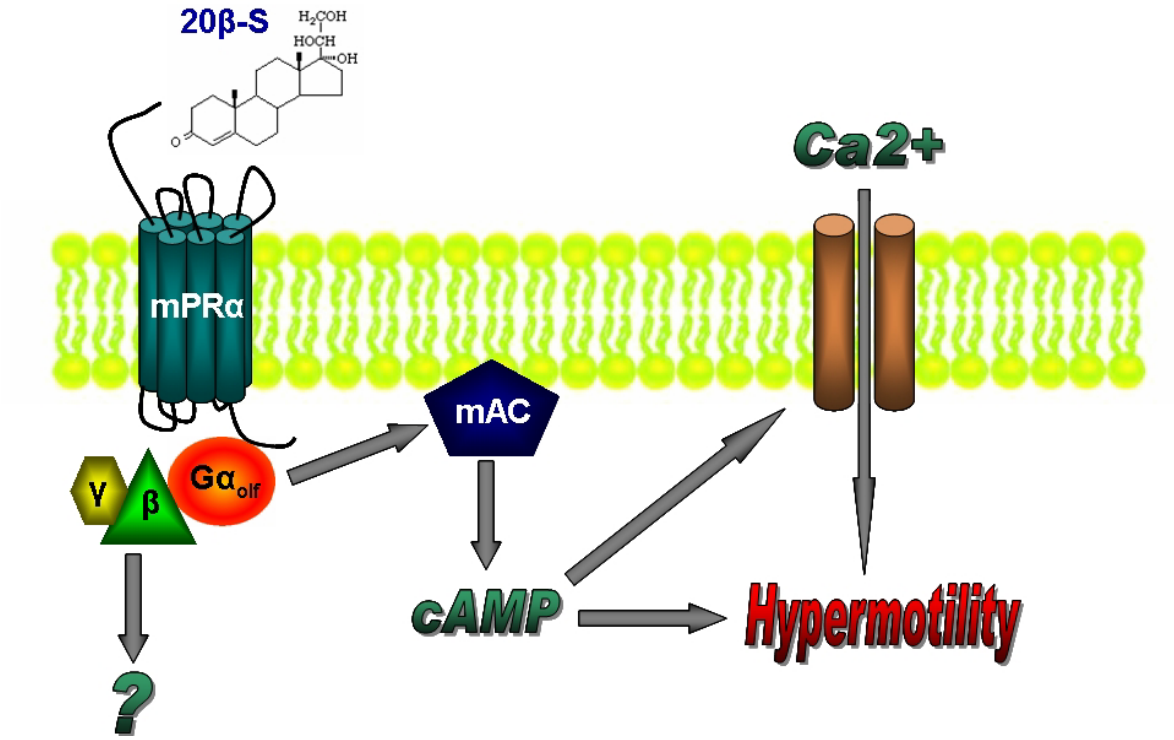

Fig. 3.7. Proposed model of 20β-S-stimulation of sperm hypermotility in the Atlantic croaker. Activation of G_{olf} proteins and membrane adenylyl cyclases (mACs), by 20β-S binding to mPRα, stimulates increases in intracellular cAMP concentrations which results in opening of cAMP-gated Ca^{2+} channels which both act to induce sperm hypermotility.

CHAPTER 4

PROGESTIN STIMULATION OF SPERM HYPERMOTILITY AND IDENTIFICATION OF mPRα ON SOUTHERN FLOUNDER

Summary

In teleosts, progestin stimulation of sperm hypermotility has been demonstrated in members of the family *Sciaenidae*. In this study, stimulation of sperm hypermotility by the teleost progestin, 20β-S, was investigated in another teleost, the southern flounder. Furthermore, the novel membrane progestin receptor alpha (mPRα) was examined as a candidate for the receptor mediating this action in flounder sperm. Treatment of flounder sperm with 20β-S increased sperm velocity in a dose-dependent manner. This effect was specific for 20β-S and E_2, 11-KT, cortisol and another teleost progestin, 17,20β-P did not alter sperm velocity. Biochemical characterization of 20β-S binding to flounder sperm membranes identified a single class of high affinity, saturable binding sites specific for 20β-S. Pre-incubation of sperm membranes with GTPγS reduced 20β-S binding, suggesting that the 20β-S receptor on sperm membranes is coupled to G-proteins. The flounder mPRα was cloned from flounder testis and shown to be similar to other teleost mPRαs. Flounder mPRα mRNA and protein were both expressed in flounder sperm and testes. In flounder sperm, expression of mPRα protein was restricted to the sperm midpiece, the region which regulates sperm motility. Taken together, results suggest that progestins stimulate sperm hypermotility in southern flounder through activation of a progestin receptor coupled to G-proteins detected on flounder sperm membranes, which

is likely mPRα. Furthermore, these findings provide the first evidence of direct actions of progestins to stimulate sperm hypermotility in a non-sciaenid teleost, suggesting that this phenomenon is wide-spread across marine fishes.

Introduction

The ability of progestins to act rapidly at the plasma membrane of sperm to stimulate sperm hypermotility and the acrosome reaction, which are necessary for successful penetration and fertilization of oocytes (Baldi et al., 1995; Luconi et al., 2004; Parinaud and Milhet, 1996; Sabeur et al., 1996; Sirivaidyapong et al., 1999; Therien and Manjunath, 2003), have been described in several mammalian species (Baldi et al., 2002; Blackmore et al., 1991; Revelli et al., 1994) . However, it is not known whether hypermotility and the acrosome reaction are controlled by similar progestin-mediated pathways (Baldi et al., 2002; Bedu-Addo et al., 2007; Ho and Suarez, 2001). In teleosts, whose sperm do not have an acrosome and do not undergo the acrosome reaction, progestin stimulation of sperm hypermotility has been shown in three members of the family *Sciaenidae*; the spotted seatrout (*Cynoscion nebulosus*), the Atlantic croaker (*Micropogonias undulatus*) and red drum (*Sciaenops ocellatus*) (Chapter 2) (Thomas, 2003; Thomas et al., 2006). High affinity binding sites have been identified for the endogenous progestin hormone 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) on croaker and seatrout sperm membranes which have the characteristics of membrane bound progestin receptors (Thomas et al., 1997; Thomas et al., 2005). However, like

mammals, the identity of these receptors and whether they are the mediator of progestin-stimulation of sperm hypermotility are currently unknown.

The recently discovered novel cDNA in the spotted seatrout encoding a putative membrane progestin receptor (mPRα) is a candidate for the receptor mediating 20β-S-stimulation of sperm hypermotility. In seatrout oocytes, mPRα has been shown to specifically bind 20β-S and function as a G-protein coupled receptor (GPCR) by activating inhibitory G-proteins (Thomas et al., 2002; Zhu et al., 2003b). The receptor is also expressed on seatrout sperm membranes (Zhu et al., 2003b), and is localized to the sperm midpiece; the mitochondrial-rich region of sperm which regulates motility (Tubbs and Thomas, *in press*). Moreover, increased mPRα expression is associated with high sperm motility in this species. The mPRα is also expressed in croaker sperm membranes and localized to the midpiece (Thomas et al., 2005). Further investigation into the signaling pathways activated by 20β-S in croaker sperm suggest that mPRα acts as a GPCR by coupling to, and activating olfactory type G-proteins (G_{olf}) (Chapter 2). Treatment with 20β-S results in activation of membrane adenylyl cyclases (mACs) to increase intracellular 3',5'-cyclic adenosine monophosphate (cAMP) concentrations, which involves activation of G_{olf} proteins. Inhibition of mAC activation blocks the ability of 20β-S to stimulate cAMP production in croaker sperm membrane preparations as well as the ability of 20β-S to stimulate sperm hypermotility (Chapter 2). These findings provide the first identification of a receptor and mechanism mediating progestin-stimulation of sperm hypermotility for any species.

Preliminary evidence in our laboratory has suggested that direct actions of progestins to stimulate sperm hypermotility in fish may extend beyond the family *Sciaenidae* (Thomas et al., 2006). Treatment of southern flounder sperm (Family: *Achiropsettidae*; *Platylicthys lethigstomata*) with 20 nM 20β-S results in a significant increase in sperm velocity. Furthermore, western blot analyses using an antibody generated against seatrout mPRα have shown the presence of an immunoreactive protein of approximately 80 kDa, which corresponds to the primary immunoreactive protein in seatrout, croaker and red drum sperm (Thomas et al., 2006). This suggests that mPRα-mediated progestin-stimulation of sperm hypermotility may be conserved among different teleost groups. Thus, the goals of the present study were to determine if progestins can stimulate flounder sperm hypermotility, investigate whether a binding moiety with the biochemical binding characteristics of a membrane progestin receptor is present on flounder sperm and determine if mPRα is a candidate for the receptor which mediates progestin stimulation of sperm hypermotility in flounder.

Materials and Methods

Chemicals

All steroids were purchased from Steraloids (Newport, RI). [^{3}H]-20β-S was made by enzymatic conversion of [^{3}H]-11-deoxycortisol (50 Ci/mmol; Amersham, Piscataway, NJ) as described previously (Scott et al., 1982). All other chemicals and reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Animals

Fully mature southern flounder were captured with nets in the Corpus Christi Shipping Channel near Port Aransas, TX during their reproductive season (Sep-Nov) and immediately transferred to tanks at the University of Texas Marine Science Institute. Flounder were maintained in 12,000 L recirculating tanks at 20ºC with a photoperiod of 11L:13D to promote and maintain gonadal development. All fish were acclimated to laboratory conditions for at least two weeks prior to use in experiments, which were performed following procedures approved by the Institutional Animal Care and Use Committee of University of Texas at Austin.

Sperm and testis collection and membrane preparation

Sperm were collected with a syringe from the cloaca of fully mature flounder by applying gentle pressure to the abdominal region and placed on ice or in liquid N_2. Contamination of milt with urine was avoided, since it can cause premature activation of sperm. For all experiments, sperm was pooled from multiple donors (n=2-3). Testes were collected after fish were humanely sacrificed by cervical dislocation. After collection, 2-3 ml sperm or 1-2 g of testes were added to 10 ml of ice-cold homogenization buffer (HAED; 25 mM HEPES, 10 mM NaCl, 10 mM $MgCl_2$, 1 mM dithioerythritol, 1 mM EDTA, pH 7.6) and passed 10 times through a glass homogenizer. Homogenates were then forced through a 23.5 gauge needle, sonicated for 6 sec on ice and centrifuged at 500 x *g* for 20 min at 4ºC to remove nuclear material. Supernatants were collected and centrifuged at 17,000 x *g* for 30 min at 4ºC. The supernatant was removed and sperm

membrane pellets were resuspended in HAED with HALT protease inhibitor cocktail (Pierce, Rockford, IL). Samples were then adjusted to a final protein concentration of ~1 mg/ml using a Bradford protein assay (Bio-Rad, Hercules, CA).

Sperm motility analyses

Sperm motility analyses were performed as described previously for Atlantic croaker using sperm from individual fish (Thomas and Doughty, 2004). After collection, sperm were diluted 100-fold in predilution solution (160 mM NaCl, 8.6 mM KCl, 0.1 mM $CaCl_2$, 10 mM $NaHCO_3$, 1.3 mM Na_2HPO_4, 5 mM D-glucose, pH 7.8). For dose-response experiments, sperm were treated with different concentrations of 20β-S (1-200 nM) or vehicle (0.01% ethanol) for 1 min in predilution solution. For steroid specificity experiments, sperm were incubated with 20 nM of steroid (17β-estradiol (E_2), 11-ketotestosterone (11-KT), cortisol, 20β-S, or 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) or ethanol (0.01%). A 2 µl aliquot of sperm was then placed on a microscope slide and diluted with 25 µl of artificial seawater (680 mOsm/kg). Slides were immediately placed under a dark field microscope and recorded using a CCD camera (Cohu Electronics, San Diego, CA) and a VHS recorder. Sperm velocity was then determined using a VP110 video digitizer and ExpertVision software (Motion Analysis Corporation, Santa Rosa, CA). For each treatment, sperm velocity was recorded at a rate of 30 frames per sec for 5 sec and the mean velocity for individual sperm (mean n=40 per treatment) was determined.

Binding of 20β-S to flounder sperm membranes

Flounder sperm membranes were isolated as described above. For saturation and Scatchard analyses, 125 µl of sperm membranes (~1 mg protein/ml) were incubated with 125 µl of increasing concentrations of [^{3}H]-20β-S (1.17-32.2 nM final concentration) in the absence (total binding) or presence (non-specific binding) of 30 µM cold 20β-S for 30 min at 4ºC with gentle shaking. Samples were then filtered through Whatman GF/B glass fiber filters using a Brandel Semi-Auto Harvester (Gaithersburg, MD) and rinsed 3 times with 5 ml of ice-cold wash buffer (25 mM HEPES, 10 mM NaCl, 1 mM EDTA, pH 7.6). The amount of [^{3}H]-20β-S binding to flounder sperm membranes was measured using a Beckman LS6000 liquid scintillation counter (Fullerton, CA). Specific binding of [^{3}H]-20β-S was calculated by subtracting non-specific binding from total binding.

For competition studies, sperm membrane preparations were incubated with 10 nM [^{3}H]-20β-S in the presence of 100 nM of various competitors (17β-estradiol, 11-KT, cortisol, 20β-S, 17,20β-P, or R5020) for 45 min at 4ºC. A set of samples was also incubated with 20 nM 20β-S or 17,20β-P. Samples were then filtered as described above and the amount of specific binding of [^{3}H]-20β-S in the presence of the various competitors was determined. The results were expressed as a percentage of [^{3}H]-20β-S binding in the absence of any steroid competitors.

To investigate the time course of dissociation of 20β-S binding to flounder sperm membranes the dissociation of [^{3}H]-20β-S in the presence of excess cold 20β-S was examined. For these experiments, flounder sperm membrane preparations were incubated in the presence of 10 nM [^{3}H]-20β-S for 20 min at 4ºC to allow 20β-S binding to reach

equilibrium. Membrane preparations were then added to tubes containing 10 μM cold 20β-S for 5, 10 and 20 min and then filtered. The dissociation of [^{3}H]-20β-S from flounder sperm membrane was then determined by calculating the percent of specific binding of [^{3}H]-20β-S after 5, 10 and 20 min in the presence of cold 20β-S compared to samples incubated with only [^{3}H]-20β-S for 20 min.

To determine if the 20β-S receptor on flounder sperm membranes is coupled to a G-protein, specific [^{3}H]-20β-S binding in the presence or absence of guanosine 5'-(3-*O*-thio)triphosphate (GTPγS) was examined as described previously (Pace and Thomas, 2005). Briefly, sperm membranes were pre-incubated with 12.5 μM GTPγS for 20 min at 18ºC. Membranes were then incubated with either 5 or 10 nM [^{3}H]-20β-S for 30 min at 4ºC. Specific binding of [^{3}H]-20β-S was determined following filtration as described above.

Cloning of southern flounder mPRα

Testes from fully mature southern flounder were collected and immediately frozen in liquid N_2. Total RNA was extracted using TriReagent (Sigma, St. Louis, MO) and DNase treated using a DNA-Free RNA kit (Zymo Research, Orange, CA). Approximately 5 μg of total RNA was used to perform 3'-rapid amplification of cDNA ends (RACE) using a GeneRacer kit following the manufacturer's instructions (Invitrogen, Carlsbad, CA). To obtain a partial sequence of flounder mPRα, a final PCR was performed using Platinum Taq HiFi master mix (Invitrogen, Carlsbad, CA) and 10 pmol of primers designed against the 5' region of seatrout mPRα (5'-

TACCGCTGCCACCACTACCTC-3') and the 3'-RACE adaptor sequence (5′-GCTGTCAACGATACGCTACGTAACG-3′). The 5' primer for seatrout mPRα was used because it is designed against a highly conserved region of mPRα, which displays >90% nucleotide homology across fish species. The cycling profile used was 30 sec at 94ºC, 30 sec at 65ºC, and 1 min at 72ºC for 35 cycles, followed by a final 5 min extension period at 72ºC. PCR products were then run on a 1% agarose gel and visualized with SYBR green. The amplified products were excised from the gel and purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA). Purified PCR products were then cloned into a pGEM-T Easy vector (Promega, Madison, WI) and transformed into competent *Escherichia coli* (XL-1 Blue; Stratagene, La Jolla, CA) following manufacturer's instructions. Bacteria were grown overnight and positive colonies were isolated and plasmids purified using a QIAprep spin miniprep kit (Qiagen, Valencia, CA). Purified plasmids were sequenced at the UT Austin DNA sequencing facility using T7 and Sp6 primers. Flounder mPRα was cloned from 2 fish and for each RACE reaction, two positive clones were sequenced in both directions using T7 or Sp6 primers. Homology and alignments of flounder mPRα to other fish mPRαs was determined using ClustalW (http://www.ebi.ac.uk/clustalw; European Bioinfomatics Institute). The accession numbers for the fish mPRα sequences used were; spotted seatrout (*Cynoscion nebulosus*: Q801D8), *Fugu* (*Takifugu rubripes*: NP_0010359), zebrafish (*Danio rerio*: NP_899188), goldfish (*Carassius auratus*: BAD06917), and channel catfish (*Ictalurus punctatus*: AAS45554).

RT-PCR

Total RNA from flounder sperm and testes was isolated as described above and reverse transcribed using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random primers following the manufacturer's instructions. PCR was performed using Platinum Taq HiFi master mix with newly generated cDNA and 10 pmol of each primer for flounder mPRα, designed from sequence information obtained from the cloning of this gene. The primers used were sense 5'-CAGCTGGCGCTACTACTTCC-3', antisense 5'-CGCTGAAGGAGAAGTAGGTG-3'. The cycling profile used was; 30 sec at 94ºC, 30 sec at 60ºC, and 1 min at 72ºC for 35 cycles, followed by a final 5 min extension period at 72ºC. PCR products were run on a 1% agarose gel and visualized using SYBR green.

Western blot analyses

Flounder sperm and testicular membrane preparations were solubilized in SDS-PAGE loading buffer (0.5 M Tris-HCl, 10% SDS, 0.5% bromophenol blue, 10% glycerol). Membrane proteins were resolved on 10% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked in buffer containing 5% non-fat dry milk and 0.1% Tween-20 in phosphate buffered saline (PBS; 136 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 10 mM KH_2PO_4, pH 7.4). Blocked membranes were rinsed three times with PBS and then incubated with a rabbit polyclonal primary antibody directed against a 15-mer peptide sequence in the N-terminal region of seatrout mPRα (YRQPDQSWRYYFLTL; 1:2000) overnight in blocking buffer at 4ºC (Thomas et al.,

2007). Membranes were then rinsed three times with PBS and incubated with a goat anti-rabbit horseradish peroxidase conjugated secondary antibody (1:5000; Abcam, Cambridge, UK) in blocking buffer for 1 hr at room temperature. Membranes were given three final rinses with PBS and proteins visualized using SuperSignal WestPico chemilumenescent substrate (Pierce, Rockford, IL).

Immunocytochemical localization of mPRα in southern flounder sperm

Flounder mPRα protein was localized on sperm as described previously for Atlantic croaker sperm with few modifications (Thomas et al., 2005). Briefly, flounder sperm were collected, spread on poly-L-lysine coated slides and air dried. Sperm were then fixed in 4% paraformaldehyde for 30 min on ice then blocked in 2% bovine serum albumin (BSA) in PBS for 1 hr at room temperature. Slides were then rinsed in PBS and incubated overnight with the seatrout mPRα antibody (1:1000) at 4ºC. To confirm antibody specificity the primary antibody was pre-absorbed against the peptide antigen (0.02 mg peptide/ml antibody) for 18 hr at 4ºC. After three rinses with PBS, slides were then incubated with a goat anti-rabbit AlexaFluor 488 secondary antibody (1:2000; Molecular Probes, Eugene, OR) for 1 hr in blocking solution. Slides were then rinsed in PBS and coverslips were added using ProLong Antifade Gold reagent (Invitrogen, Carlsbad, CA). Sperm were visualized using a Nikon E600 fluorescent microscope.

20β-S radioimmunoassay

Flounder milt was collected and placed immediately on ice. To separate sperm from seminal plasma, milt samples were centrifuged at 14,000 x *g* for 10 min at 4ºC. 100 µl of seminal plasma was then collected, steroids extracted and the concentration of 20β-S in southern flounder seminal fluid was determined in duplicate samples using a radioimmunoassay protocol previously described for Atlantic croaker (Mathews et al., 2002; Trant and Thomas, 1989).

Statistical analyses

For all experiments, data are presented as means ± SEM. Statistical significance was determined using a Student's t-test or one-way ANOVA and Dunnett's multiple comparison post-test using GraphPad Prism Software (San Diego, CA).

Results

Progestin stimulation of flounder sperm hypermotility

Treatment of flounder sperm from 3 individual fish with 20β-S resulted in an increase in sperm velocity in a dose-dependent manner (Fig. 4.1A). Treatment with artificial seawater alone (control groups) resulted in sperm velocity of 119.9 ± 3.2 µm/sec (n=43). However, treatment with 5 nM 20β-S significantly increased sperm velocity to 139.4 ± 2.9 µm/sec ($p<0.05$; n=37). This resulted in a maximal activation of flounder sperm as 20, 100 and 200 nM did not further increase sperm velocity above that of the 5 nM dose. In another experiment, only 20β-S was effective in increasing flounder sperm

velocity at a concentration of 20 nM (mean velocity=153.8 ± 3.8 μm/sec; $p<0.05$; n=55) compared to control samples (mean velocity=130.1 ± 5.3 μm/sec; n=28; Fig. 4.1B). Incubation of flounder sperm with 20 nM of 11-KT, 17β-estradiol or cortisol did not significantly affect flounder sperm velocity. Furthermore, treatment with 20 nM of the other major teleost progestin, 17,20β-P, did not significantly alter sperm velocity compared to control treatments (mean velocity=119.3 ± 4.7 μm/sec; n=28).

Binding of 20β-S to flounder sperm membranes

Saturation and Scatchard analyses demonstrated the presence of a single class of saturable, high affinity, limited capacity 20β-S binding sites (Fig. 4.2A, B). The mean K_d and the B_{max} of the 20β-S receptor on flounder sperm membranes were 22.95 ± 6.6 nM and 0.013 ± 0.05 nM, respectively (n=4). Dissociation experiments showed that total dissociation of 20β-S is rapid, with a predicted dissociation half time of 1.5 minutes. Furthermore, incubation of flounder sperm with excess cold 20β-S resulted in complete dissociation of [^{3}H]-20β-S from flounder sperm membranes at both 5 and 10 minutes (Fig. 4.2C). Competition studies confirmed that the receptor on flounder sperm membranes is specific for [^{3}H]-20β-S, since 100 nM cortisol, 17β-estradiol, 11-KT and the nuclear progestin receptor agonist, R5020, did not significantly displace 10 nM [^{3}H]-20β-S (Fig. 4.2D). Treatment of sperm membranes with 20 nM of cold 20β-S did not result in a significant displacement of [^{3}H]-20β-S, but was 75% of control binding. Incubation with of another teleost progestin, 17,20β-P did not significantly displace [^{3}H]-20β-S. Although 100 nM 17,20β-P also did not significantly displace [^{3}H]-20β-S from

flounder sperm membranes, its displacement was the highest of any other steroid tested, which was approximately half that of (~49%) 100 nM cold 20β-S, and (Fig. 4.2D). Finally, pre-incubation of flounder sperm membranes with 12.5 μM GTPγS resulted in a significant decrease in [^{3}H]-20β-S binding, which was approximately half that of control samples. This trend was observed for both concentrations of [^{3}H]-20β-S tested (Fig. 4.3).

Cloning of Southern flounder mPRα

A single PCR product encoding a partial sequence of flounder mPRα was cloned from flounder testes following 3'-RACE. Since the final PCR of 3'-RACE products was performed using primers directed against the start codon of seatrout mPRα, the identity of the first 21 bp of flounder mPRα was not obtained. However, this region of mPRα is highly conserved at both the nucleotide (>90%) and amino acid (>99%) level in teleosts, and is likely similar in flounder. The flounder specific mPRα open reading frame sequence is predicted to be composed of 1059 base pairs which display approximately 90% and 98% nucleotide and amino acid identity, respectively, to seatrout mPRα (Fig. 4.4).

The flounder mPRα open reading frame nucleotide sequence was more similar to mPRα of other members of superorder *Acanthopterygii*, seatrout (91%) and *Fugu* (84%), than mPRα sequences in zebrafish (74%), goldfish (74%) and channel catfish (73%), which are members of the superorder *Ostariophysi*. In *Ostariophysians*, known mPRα proteins are 354 amino acid residues, whereas mPRαs of *Acanthopterygians* are comprised of 352 amino acid residues due to a probable deletion of six nucleotides

approximately 700 bp from the start codon. This results in two fewer amino acids in the 3rd extracellular loop of mPRα in *Ostariphysians*. Other predicted amino acid residues that differed between the two groups were 57 (Q/P), 61 (S/N), 124 (F/C), 165 (A/V), 177 (L/F), 252 (H/Q), 265 (C/Y), and 271 (L/W). The 15 amino acid region of seatrout mPRα used to generate the polyclonal antibody used in this study differed from flounder mPRα at only one position (Fig. 4.4).

Expression of mPRα in flounder sperm and testes

RT-PCR and western blot analyses demonstrate that mPRα mRNA and protein are expressed in both flounder sperm and testes (Fig. 4.5A, B). Using internal primers designed against flounder mPRα, an amplicon of predicted size (200bp) was detected following RT-PCR. Western blot analyses revealed the presence of both 40 kDa and 80 kDa proteins in testes and an 80 kDa band in sperm. These bands are thought to correspond to mPRα monomers and dimers, respectively, and are the major bands found in sperm and testis of two other fish species, the Atlantic croaker and spotted seatrout (Thomas et al., 2007; Thomas et al., 2005).

Localization of mPRα in flounder sperm

Immunocytochemical analyses demonstrate that mPRα is localized to the midpiece of flounder sperm. No staining was evident on sperm heads and weak staining for mPRα was present on the sperm flagella (Fig. 4.6).

Presence of 20β-S in flounder seminal fluid

20β-S was detected in seminal plasma of milt in all fish examined using an RIA validated for this steroid in Atlantic croaker The mean concentration of 20β-S flounder milt was 0.697 ± 0.23 ng 20β-S/ml of seminal plasma (n=4).

Discussion

To date, progestins have been shown to regulate teleost sperm motility by two different mechanisms. One mechanism, described in the Japanese eel and masu salmon (Miura et al., 1991; Miura et al., 1992) involves progestins acting on the sperm duct to increase seminal fluid pH which provides sperm the potential for motility. The other proposed mechanism, which has been described in members of the family *Sciaenidae*, involves a direct action of progestins on sperm, presumably through membrane-bound receptors to rapidly stimulate sperm hypermotility. In the present study, this second mechanism was extended beyond sciaenids by demonstrating the direct actions of 20β-S on southern flounder sperm (Family: *Achiropsettidae*) to increase sperm velocity. Furthermore, a progestin binding moiety was identified on flounder sperm membranes that is specific for 20β-S and has the characteristics of a membrane progestin receptor coupled to G-proteins. This receptor is suggested to be the novel membrane progestin receptor alpha (mPRα) which is shown to be expressed in flounder sperm and testes and is localized to the sperm midpiece, which is the region responsible for the regulation of sperm motility. Taken together, these findings suggest that progestin stimulation of sperm

hypermotility through mPRα may be widespread among *Acanthopterygiian* fishes, including perciforms and flatfishes.

The actions of 20β-S to increase sperm motility in flounder corroborate previous findings in croaker, in which 20β-S increased sperm motility in a dose-dependent manner (Thomas, 2003). The lowest concentrations of 20β-S to significantly increase sperm velocity (5 and 10 nM) are similar in the two species and fall within the range of circulating plasma 20β-S levels in male croaker, and are likely of physiological relevance in terms of regulating sperm motility while sperm reside in the testis and sperm duct in this species (Thomas, 2003). In flounder, 20β-S is also synthesized *in vivo* and is present in milt in concentrations similar to progestins in at least one other species of teleost, the Atlantic salmon (King and Young, 2001). Thus, 20β-S likely acts on flounder sperm while they reside in the sperm duct. Another possibility, however, is that during spawning, 20β-S concentrations released in ovarian fluid following ovulation are sufficient to increase sperm velocity in these two species. A similar mechanism is suggested for progesterone signaling to sperm in mammals (Jaiswal et al., 1999; Teves et al., 2006) and ovarian fluid has also been shown to stimulate sperm motility in some teleost species (Lahnsteiner, 2002; Litvak and Trippel, 1998). Although an increase in 20β-S production prior to ovulation occurs in croaker, in which 20β-S is the oocyte maturation inducing steroid (MIS) (Patino and Thomas, 1990), it is not clear whether this occurs in southern flounder, in which the identity of the MIS is not known. However, this steroid is produced in male southern flounder and in other flatfishes, 20β-S can induce oocyte maturation (Canario and Scott, 1990) and is produced *in vivo* in at least one

species, the turbot (Mugnier et al., 1997). Thus, production of 20β-S by female southern flounder is likely, and might also be present in ovarian fluid released during spawning in concentrations sufficient to increase flounder sperm velocity.

Biochemical characterization of 20β-S binding to flounder sperm membranes identified a single class of receptors which are saturable, bind 20β-S with high affinity and have limited capacity, which are characteristics typical of membrane progestin receptors. Furthermore, the binding characteristics of the 20β-S receptor on flounder sperm membranes are similar to 20β-S receptors on sperm membranes in other teleosts. The dissociation constant (K_d) of the receptor on flounder sperm membranes was 22.95 nM, and corresponds to those of the receptors on seatrout and croaker sperm membranes which are 22.17 and 25.5 nM, respectively (Thomas et al., 1997; Thomas et al., 2005). In addition, the mean concentration of progestin receptors was 0.013 nM which is similar to receptor concentrations on seatrout and croaker sperm membranes (0.00261 nM and 0.084 nM) (Thomas et al., 1997; Thomas et al., 2005). For all of these species, the receptor characteristics are similar to recombinant seatrout mPRα which was recently shown to have a K_d of 7.58 nM and a B_{max} of 0.026 nM (Thomas et al., 2007). The receptor on flounder sperm membranes was also specific for progestins, as binding of 20β-S was not significantly reduced in the presence of 10-fold excess of cortisol, 17β-estradiol or 11-KT, and the nuclear progestin receptor agonist, R5020, whereas 10-fold excess of the other major teleost progestin, 17,20β-P, was an effective competitor for the receptor. Spotted seatrout and Atlantic croaker sperm membrane progestin receptors have similar steroid specificities with the highest affinity for 20β-S and moderate affinity for

17,20β-P, and little affinity for cortisol, 17β-estradiol and 11-KT (Thomas et al., 1997; Thomas et al., 2005). The rapid dissociation of 20β-S from flounder sperm membranes, which occurred in 5 min, is also consistent with findings using seatrout and croaker sperm membrane preparations, as well as recombinant seatrout mPRα from which complete dissociation of 20β-S also occurs by 5 min (Thomas et al., 1997; Thomas et al., 2007; Thomas et al., 2005). In contrast, complete dissociation of 20β-S from seatrout nuclear PR occurs after 12 hr (Pinter and Thomas, 1995) which suggests that the receptor present on flounder sperm membranes is not a nuclear progestin receptor. The ability of the non-hydrolyzable GTP analog, GTPγS, to reduce 20β-S binding to flounder sperm membranes suggests that the 20β-S receptor is coupled to a G-protein. Activation of G-proteins in plasma membranes with GTPγS, reduces the pool of available receptors coupled to G-proteins, thereby decreasing the amount of ligand that can be bound (Birnbaumer et al., 1990; Orchinik et al., 1992). This decrease in receptor binding has previously been shown in seatrout oocytes and with recombinant seatrout mPRα (Pace and Thomas, 2005; Thomas et al., 2007). Taken together, these data suggest that a membrane steroid receptor specific for progestins and coupled to a G-protein is present on flounder sperm membranes, and possesses similar characteristics to receptors on the sperm membranes of other teleost species.

A strong candidate for the receptor localized to flounder sperm membranes which regulates progestin stimulation of sperm hypermotility is mPRα. As mentioned above, the binding characteristics of the 20β-S receptor on flounder sperm membranes are similar to recombinant and native mPRαs. Although earlier studies in our laboratory demonstrated

the presence of an immunoreactive protein on flounder sperm membranes using an antibody directed against seatrout mPRα (Thomas et al., 2006), it was not known whether mPRα mRNA and protein are expressed in flounder. Here we report that flounder mPRα mRNA is present in flounder testes and sperm and shares a high degree of homology to other teleost mPRα's. In particular, flounder mPRα is more similar to seatrout and *Fugu* mPRα than to mPRα in zebrafish, goldfish and channel catfish. This supports the evolutionary history of these fishes since zebrafish, channel catfish and goldfish belong to the superorder *Ostariophysi* and the more derived flounder, seatrout and *Fugu* belong to the superorder *Acanthopterygii*. Moreover, in zebrafish and goldfish, 17,20β-P is the MIS and binds mPRα with high affinity (Tokumoto et al., 2006; Zhu et al., 2003b), whereas in seatrout, mPRα has the highest binding affinity for 20β-S (Zhu et al., 2003b), suggesting this divergence in mPRα structure influences the steroid binding properties of this receptor. The peptide sequence of seatrout mPRα used to generate the polyclonal antibody used in the present study only differs from the predicted peptide sequence of flounder mPRα by one amino acid. Thus, it is highly likely that the protein bands recognized by this antibody in flounder sperm membranes are mPRα, which is supported by the presence of 40 and 80 kDa proteins in flounder sperm membranes that correspond to results with seatrout and croaker sperm and testes (Thomas et al., 2005). These protein sizes probably correspond to mPRα monomers and dimers commonly found in sperm membranes (Thomas et al., 2006; Thomas et al., 2005), and other mPRα expressing cell types (Thomas et al., 2007). Furthermore, mPRα is localized to the midpiece of flounder sperm, which is consistent with its proposed role in the regulation of sperm

hypermotility. Taken together, these data clearly demonstrate that mPRα is expressed on flounder sperm membranes and localized to the midpiece establishing it as a strong candidate as the receptor which mediates 20β-S-stimulated sperm hypermotility.

Although the mechanism by which 20β-S increases flounder sperm velocity is unknown, the results of the present study suggest that the receptor to which 20β-S binds is coupled to G-proteins. In croaker sperm, the signaling pathway activated by 20β-S to stimulate sperm hypermotility has recently been shown to involve activation of olfactory G-proteins (G_{olf}) by this steroid which results in an increase in cAMP production via activation of membrane adenylyl cyclases (mACs) (Chapter 2). Furthermore, mPRα was shown to co-localize to the sperm midpiece and physically associate with G_{olf} proteins, suggesting that this receptor mediates progestin stimulation of sperm hypermotility in croaker (Chapter 2). Thus, it is likely that a similar mPRα/G_{olf}/mAC pathway is activated by 20β-S in flounder sperm and is currently under investigation.

Notably, mPRα is also present in human and mouse testes (Thomas, 2004; Zhu et al., 2003a), and in human sperm is also localized to the midpiece (Thomas and Tubbs, *unpublished obs*.). In some mammalian species, progesterone is known to induce both sperm hypermotility and the acrosome reaction. Other receptors suggested to mediate these processes include the classical progesterone receptor B (Gadkar et al., 2002; Shah et al., 2005) and a novel putative membrane localized progesterone receptor, PRMC1, originally cloned from porcine liver (Buddhikot et al., 1999; Meyer et al., 1996). However, both of these receptors have been localized to the sperm head and acrosome and not the midpiece (Buddhikot et al., 1999; Gadkar et al., 2002; Shah et al., 2005).

Thus, their roles in sperm motility are unclear. On the other hand, mPRα has been localized to the midpiece of teleost and human sperm, suggesting a direct role of this receptor in progestin stimulation of sperm hypermotility. Teleosts do not undergo the acrosome reaction since they do not have an acrosome, so they permit the examination of direct actions of progestins on sperm to regulate motility, without the potential complication of progestin activation of the acrosome reaction. Thus, results of the present study, as well as those with other teleosts and human sperm strongly suggest that mPRα mediates progestin stimulation of sperm hypermotility, and not the acrosome reaction.

In conclusion, we have shown that the progestin 20β-S can stimulate sperm hypermotility in the southern flounder, which is the first such evidence in a non-sciaenid teleost species. Furthermore, a receptor on flounder sperm membranes was biochemically characterized and has similar characteristics to the recently discovered mPRα in the spotted seatrout. Thus, it is proposed that the receptor present on flounder sperm membranes that mediates 20β-S-stimulated sperm hypermotility is mPRα, which in the present study was cloned from flounder testes and shown to be localized to the plasma membrane and midpiece of flounder sperm. Taken together, these findings demonstrate that progestin stimulation of sperm hypermotility extends beyond members of the teleost family *Sciaenidae* and suggests a widespread role of mPRα in mediating this phenomenon in advanced teleosts.

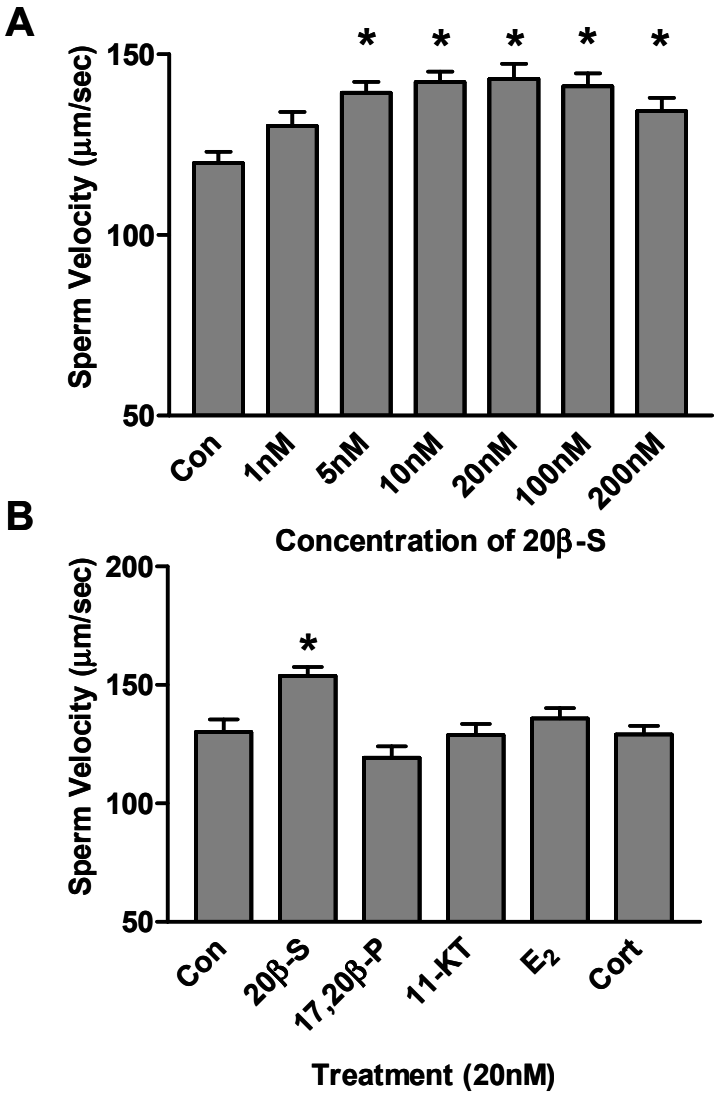

Figure 4.1. Progestin stimulation of southern flounder sperm hypermotility. (A) Effects of increasing concentrations of 20β-S (1-200 nM) on flounder sperm velocity. (B) Specificity of stimulation of flounder sperm velocity by various steroids (20 nM). Data represent mean ± SEM. Each experiment was performed in triplicate with sperm from 3 individual donors. Statistically significant differences from control samples (*$p<0.05$) were determined using a one-way ANOVA and Dunnett's post-test (n=28-55).

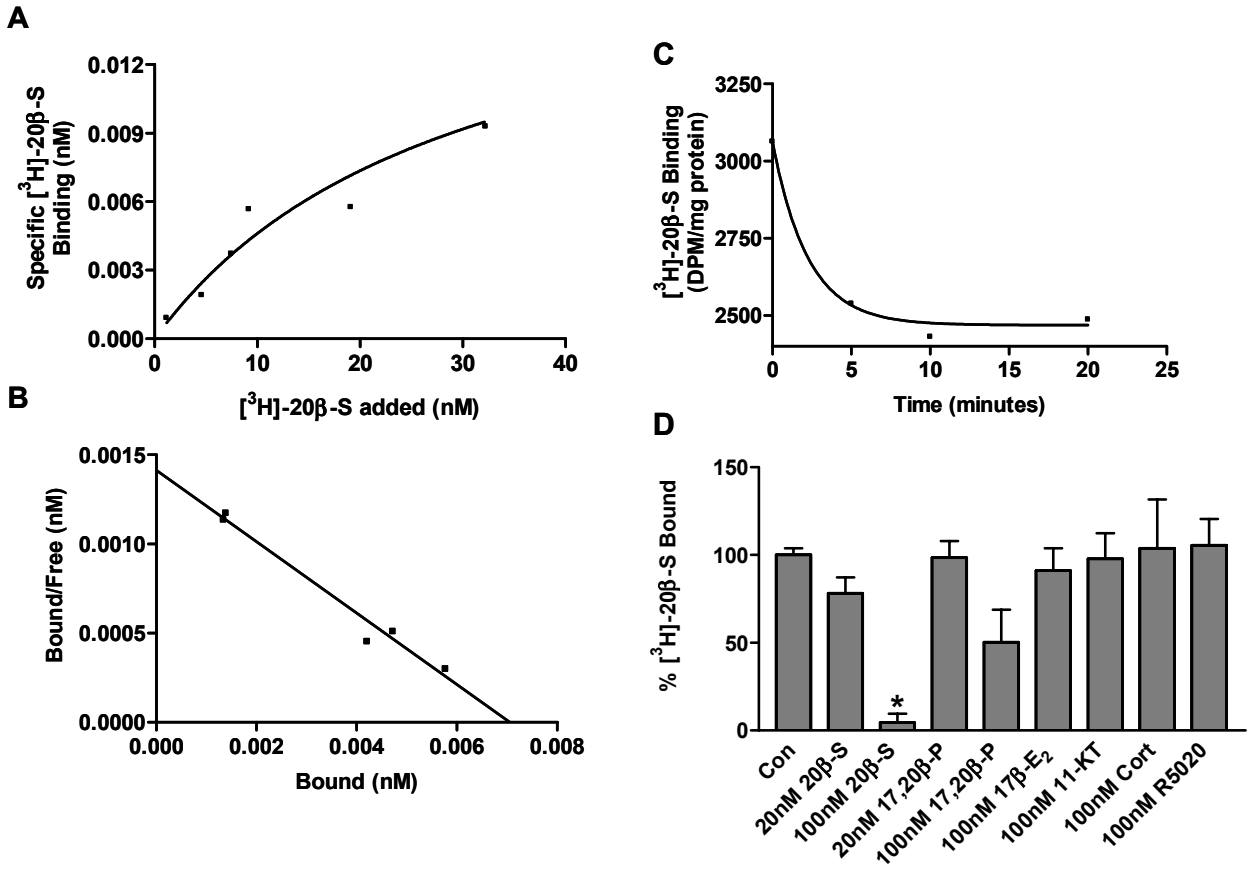

Figure 4.2. Characteristics of 20β-S binding to southern flounder sperm membranes. (A) Representative saturation plot and (B) Scatchard plot of specific [^{3}H]-20β-S binding; mean K_d 22.95 ± 6.6 nM and B_{max} 0.013 ± 0.005 nM. Experiments were repeated 4 times. (C) Representative dissociation time course of specific [^{3}H]-20β-S binding. Experiments were repeated 3 times. (D) Single point competition assays by 100 nM of various steroids for the 20β-S receptor. The teleost progestins 20β-S and 17,20β-P were also tested at 20nM. Data represent mean ± SEM. Statistically significant differences from control samples, with no non-radiolabeled steroids added, (*$p<0.001$) were determined using a one-way ANOVA and Dunnett's post-test (n=7).

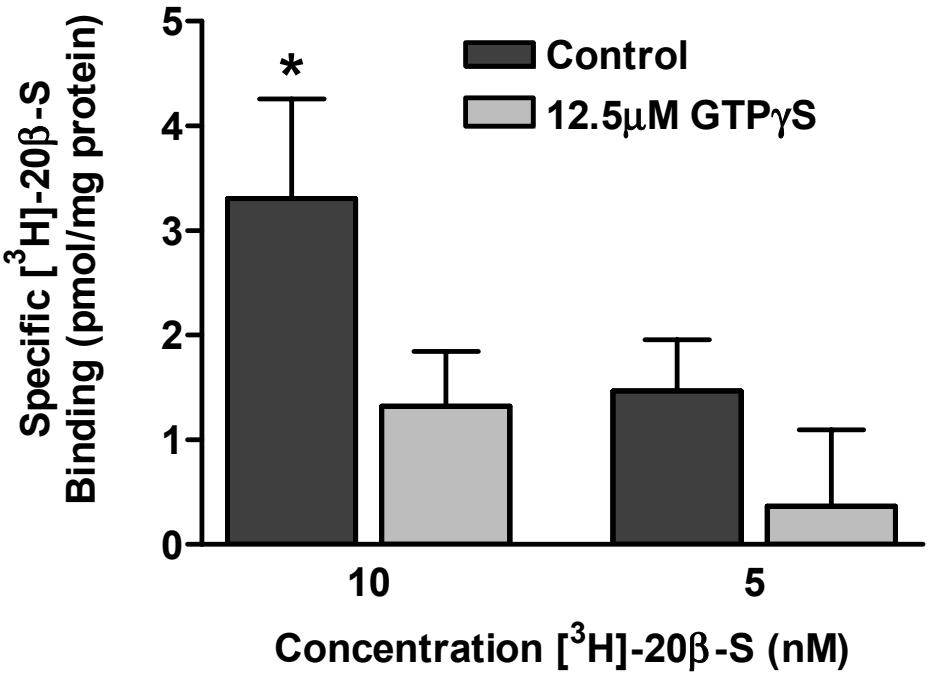

Figure 4.3. Coupling of G-proteins to the 20β-S receptor on southern flounder sperm membranes. Specific [^{3}H]-20β-S binding to flounder sperm membranes was determined following pre-incubation of flounder sperm membranes with or without (control) 12.5 μM GTPγS. Data represent mean ± SEM. Statistically significant differences from control samples (*$p<0.05$) were determined using a Student's t-test for each concentration of [^{3}H]-20β-S tested (n=5).

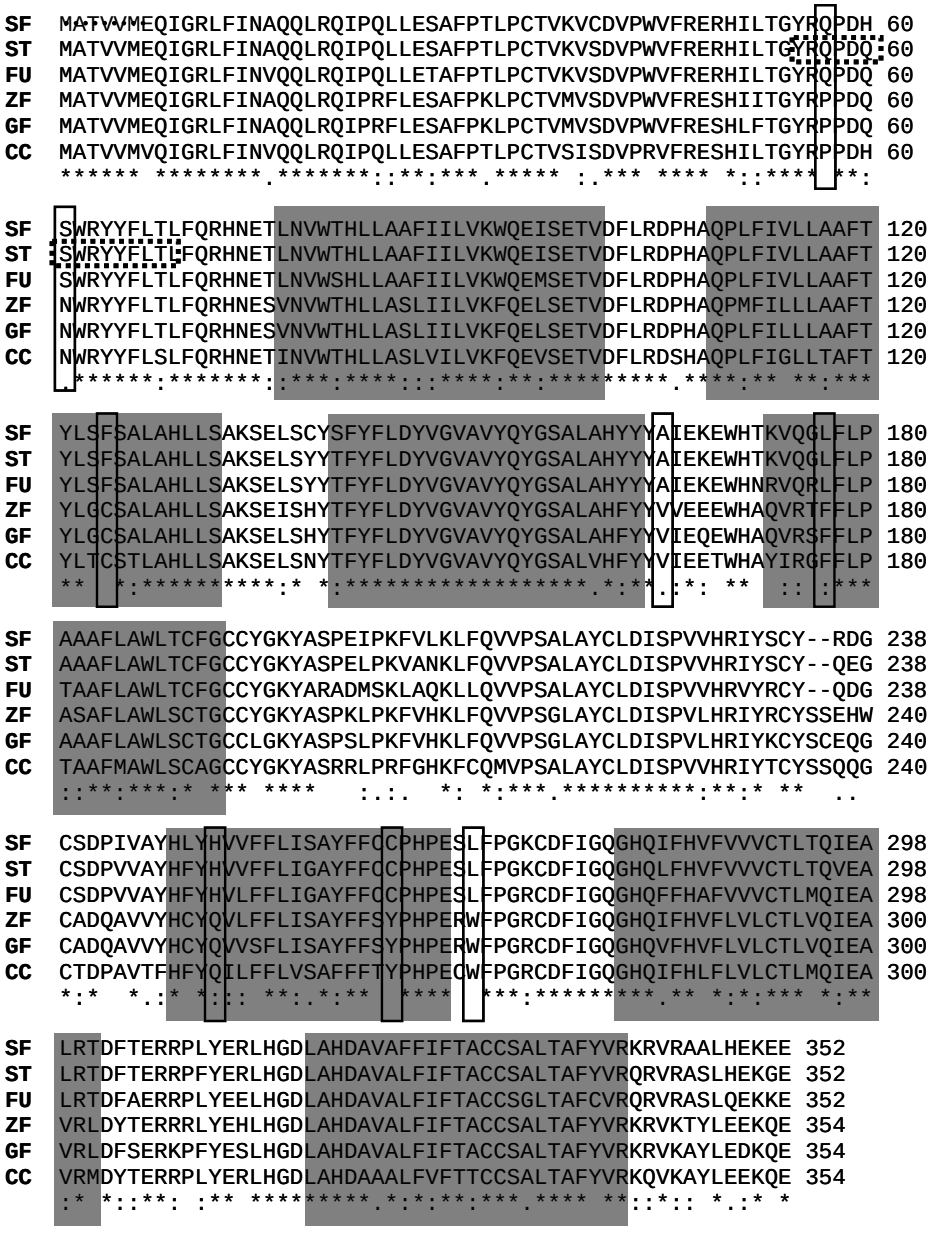

Figure 4.4. Deduced amino acid sequence of southern flounder mPRα. Alignment of flounder mPRα (SF) with mPRα sequences from different teleost species; spotted seatrout (ST), *Fugu* (FU), zebrafish (ZF), goldfish (GF) and channel catfish (CC). Shaded and solid lined boxes represent putative transmembrane domains, and divergent amino acid residues, respectively. Dashed box denotes region of seatrout mPRα used to generate the polyclonal antibody used in this study.

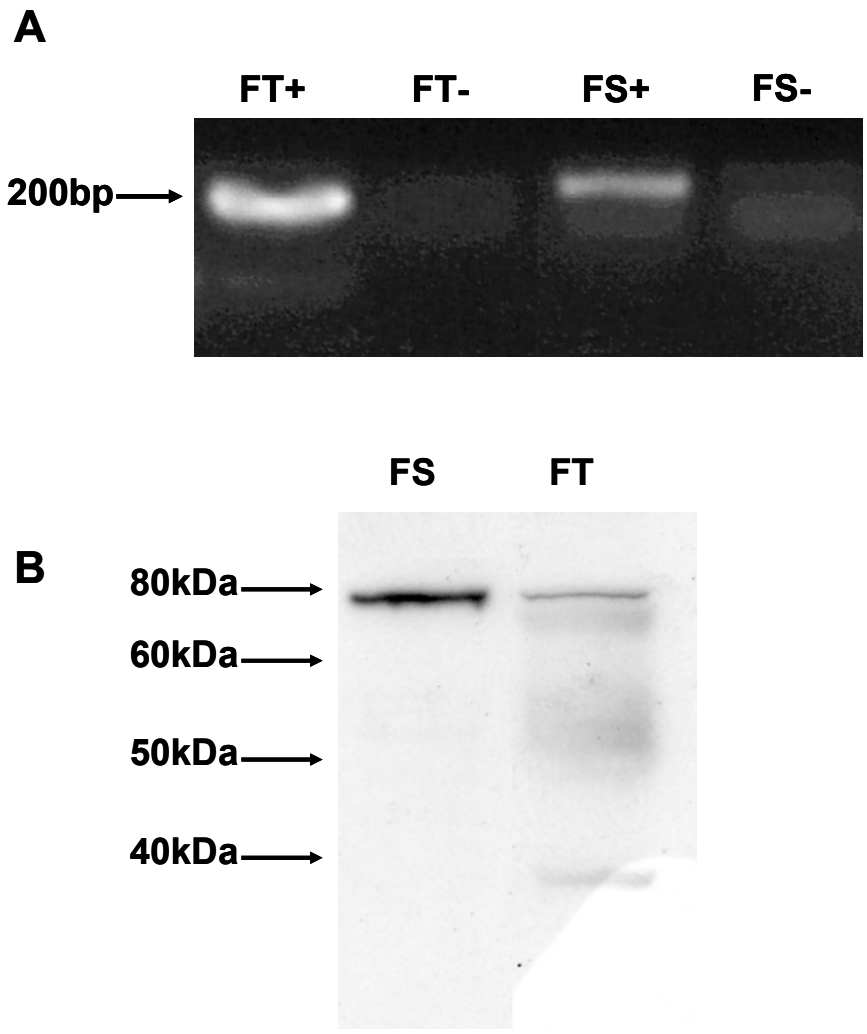

Figure 4.5. Expression of mPRα in southern flounder testis and sperm. (A) RT-PCR analyses of total RNA isolated from testes (FT+) and sperm (FS+). Reverse transcription reactions were run without reverse transcriptase (FT- and FS-) to control for genomic contamination. (B) Western blot analyses of mPRα expression on testicular (FT) and sperm (FS) membranes using a seatrout mPRα primary antibody (1:2000). Gel loading=10 μg total protein. Results typical of three experiments.

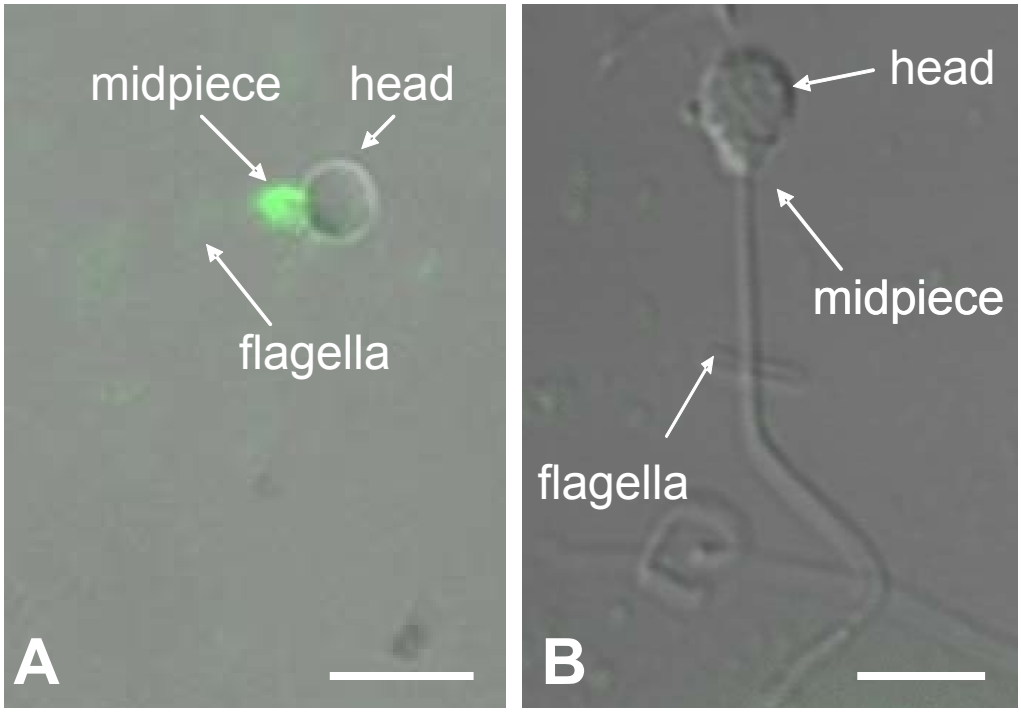

Figure 4.6. Localization of mPRα in southern flounder sperm. Immunocytochemical analyses of mPRα expression using the (A) seatrout mPRα primary antibody alone (1:1000) or (B) the antibody pre-absorbed against the peptide antigen (0.02mg peptide/ml antibody) and an AlexaFluor 488 secondary antibody (1:2000). Bar represents 5 μm. Experiments were repeated three times.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Results of the present studies clearly support the overall hypothesis that mPRα is the mediator of progestin-stimulation of sperm hypermotility in the Atlantic croaker and the likely mediator of this phenomenon in southern flounder. Based on the results of these studies, a novel mechanism involving a membrane progestin receptor, mPRα, to regulate progestin-stimulation of sperm hypermotility is proposed. In this mechanism, 20β-S binds mPRα and activates G_{olf} proteins and mACs resulting in an increase in intracellular cAMP concentrations, which in turn induces sperm hypermotility. By demonstrating that 20β-S can activate G_{olf} proteins, this study provides a unique example of hormonal activation of G-proteins and advances our understanding of the non-classical repertoire by which steroids can transduce intracellular signals in target cells.

These findings also provide strong evidence that mPRα is an important intermediary in 20β-S-stimulated sperm hypermotility in croaker and suggest a similar mechanism occurs in a non-sciaenid teleost species. Although such a mechanism has been suggested in mammalian sperm, the results are often difficult to interpret since progestins also stimulate the acrosome reaction in these species. In contrast, teleost sperm lack an acrosome and do not undergo this process, and thus provide an excellent model to examine progestin-stimulation for sperm hypermotility in isolation from the acrosome reaction. Thus, the role of mPRα in progestin-stimulation of sperm hypermotility, and not the acrosome reaction, is suggested to be an important mechanism in teleosts, since this

receptor likely mediates this phenomenon in two distantly related fishes. These finding are supported by preliminary studies in which mPRα has been shown to be localized to the human sperm midpiece, but not the acrosome. However, further investigation is necessary to definitively establish the evolutionary significance of mPRα as the receptor which mediates progestin-stimulation of sperm hypermotility.

These studies clearly establish that low nanomolar concentrations of 20β-S, which can be present in the testis can stimulate sperm hypermotility in croaker and flounder, although the physiological relevance of this phenomenon is unknown. It is likely that *in vivo*, seminal plasma 20β-S concentrations in males are sufficient to bind mPRα on sperm which then exhibit increased sperm motility when they are released into the seawater during spawning. However, it is also possible that sufficient 20β-S is present in ovarian fluid to stimulate sperm hypermotility, which is released with oocytes during spawning. Additional studies are required to test these hypotheses and to determine the physiological importance of mPRα in progestin-stimulation of sperm hypermotility.

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